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REVUE MENSUELLE DES SCIENCES PURES ET APPLIQUÉES
MONATSSCHRIFT FÜR DAS GESAMTE GEBIET DER NATURWISSENSCHAFT
RIVISTA MENSILE DI SCIENZE PURE E APPLICATE
MONTHLY JOURNAL OF PURE AND APPLIED SCIENCE

Editores: P. HUBER, Basel · R. MATTHEY, Lausanne · A. V. MURALT, Bern · L. RUZICKA, Zürich

Redactor: H. MISLIN, Basel · Mainz

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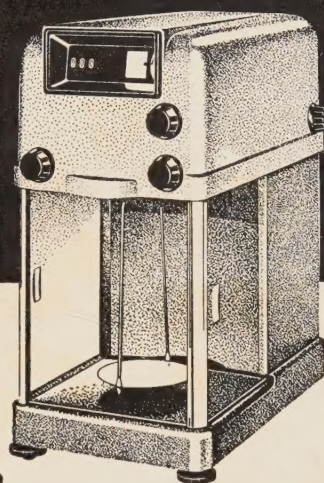
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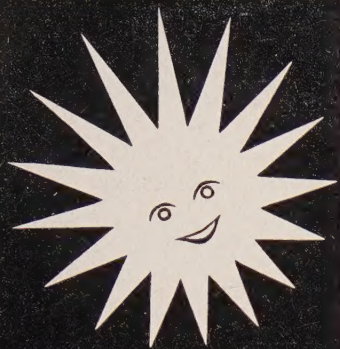
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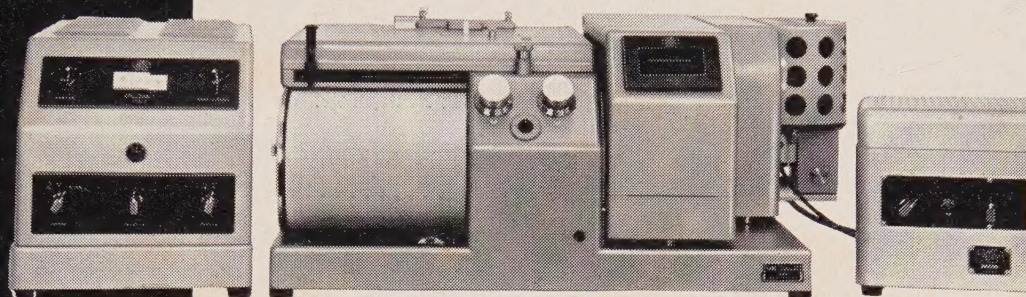
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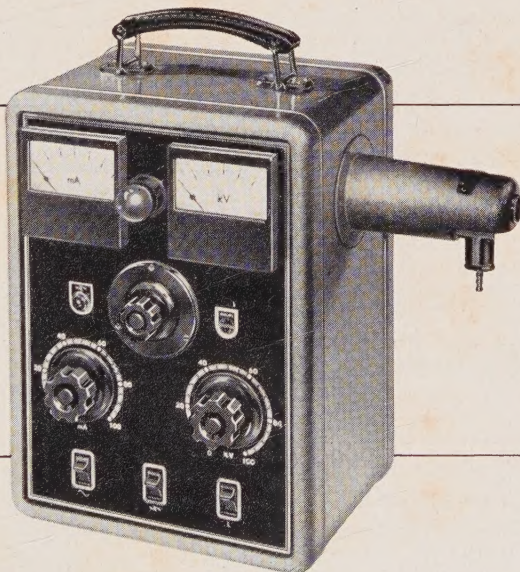
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HELVETICA PHYSICA ACTA SUPPLEMENTUM VI

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Relationships Between the Chemical Structures and the Biological Properties of the Posterior Pituitary Hormones and their Synthetic Analogues

By R. A. BOISSONNAS*, ST. GUTTMANN*, B. BERDE**, and H. KONZETT**

During the past five years, the chemistry and the pharmacology of peptides related to the posterior pituitary hormones have aroused considerable interest. In no other field of biologically active peptides have so many analogues and homologues been synthesized and submitted to such an extensive pharmacological testing.

In an attempt to find some relationships between the chemical structures of these compounds and their biological properties, we have drawn up a series of Tables which permit a systematic study of the pharmacological changes brought about by limited modification of the structure of the natural hormones, oxytocin and lysine-vasopressin.

In addition to the name and the abbreviated chemical formula of the compounds presented for comparison in the Tables, we have, in most cases, indicated the detailed chemical structure of the grouping by which the compounds in a given Table differ among themselves. We should emphasise the fact that the Tables include only chemically pure peptides, which have been demonstrated by counter-current distribution, paper chromatography and paper electrophoresis to be homogenous. They have been assayed by pharmacological methods generally used in quantitative studies on neurohypophysial hormones: contraction of the isolated rat uterus¹ and of the cat uterus *in situ*²; fall in chicken blood pressure^{3,4}; pressure increase in the lactating mammary gland of the rabbit⁵⁻⁷; rise in blood pressure of the rat⁸ and spinal cat⁹; and inhibition of diuresis in the rat¹⁰⁻¹². The quantitative results of these assays are reported in international units (IU) in the Tables. When available, the standard deviations of the assays are also indicated.

In the right-hand column of the Tables, references to papers on the chemistry and pharmacology of the synthetic analogues are cited. Most of these analogues have been synthesized and tested in our own laboratories. However, we have also included the analogues synthesized by DU VIGNEAUD or his co-workers and tested in the laboratories of DU VIGNEAUD or of VAN DYKE¹³, since for these compounds extensive and accurate chemical and pharmacological data are available.

Some other analogues have been synthesized by other research groups, but the lack of adequate chemical or pharmacological data has prevented us from including them in our Tables.

Examination of the data reported in the Tables calls for the following comments and conclusions.

Variations in Position 3. The natural posterior pituitary hormones differ among themselves by variations in positions 3 and 8 (Figure and Table I).

Systematic variation of the amino acid in position 3 was relatively easy to accomplish by following the general pattern of synthesis used for our synthesis of oxytocin¹⁴. Replacement of isoleucine by valine yielded the first analogue, Val³-oxytocin. The latter differs from oxytocin only by the absence of a $-CH_2-$ group from one of the six side-chains of the molecule (Table II). Pharmacological assays showed that the biological qualities characteristic of the oxytocin molecule are not equally affected by this small structural modification. On the isolated rat uterus and on the blood pressure of the chicken, Val³-oxytocin is only about one-eighth as potent as oxytocin. However, on

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¹ P. HOLTON, Brit. J. Pharmacol. 3, 328 (1948).

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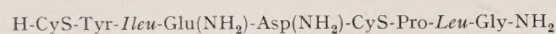
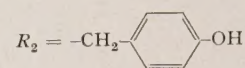
¹² J. H. BURN, Quart. J. Pharm. 4, 517 (1931).

¹³ See e.g. H. B. VAN DYKE: Vasopressins. XXII Internat. Congr. of Physiol. Sci., Buenos Aires 9–15. 8. 1959. Symposia and Special Lectures, p. 61–70.

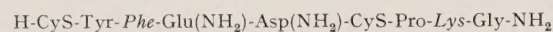
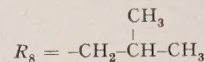
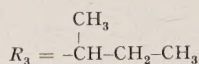
¹⁴ R. A. BOISSONNAS, ST. GUTTMANN, P.-A. JAQUENOUD, and J.-P. WALLER, Helv. chim. Acta 38, 1491 (1955).

Tab. I. Posterior pituitary hormones occurring in nature

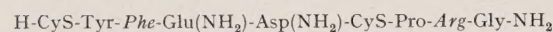
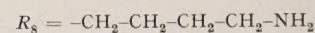
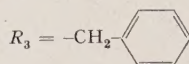
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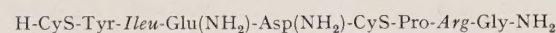
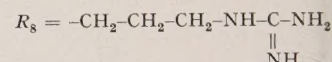
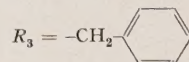
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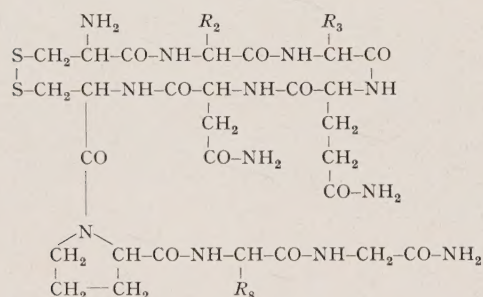
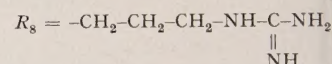
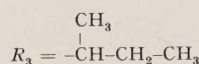
Lysine-vasopressin



Arginine-vasopressin



Arginine-vasotocin



General chemical formula of the posterior pituitary hormones.

the rabbit mammary gland and on the cat uterus *in situ*, it is still roughly half as active as oxytocin, and a similar level of activity has been observed in clinical tests in man^{15,16}. Furthermore, this slight modification of structure reduces the low pressor and antidiuretic activities, inherent in the oxytocin molecule, to about one-tenth of their former values. Thus Val³-oxytocin, while having a lower specific activity (i.e. activity per mg) than oxytocin, is actually a more selective oxytotic agent, since for a given uterine response, the clinically undesirable pressor and antidiuretic activities are less. In a manner of speaking, therefore, Val³-oxytocin is an improvement on its natural prototype.

By replacing the isoleucine of oxytocin by leucine, we obtained *Leu³-oxytocin* which differs from the natural hormone only in that the methyl group has been shifted along the side-chain at position 3. This change reduced the oxytotic activity to less than a quarter of that of the natural hormone.

The replacement of isoleucine by phenylalanine, which is present in the vasopressins in position 3, was investigated independently by our group and by

KATSOYANNIS in DU VIGNEAUD's laboratory. This modification results in an analogue (*Phe³-oxytocin* or *oxypressin*) exhibiting biological properties both of oxytocin and of the vasopressins. Strangely enough the antidiuretic activity of this compound is about ten times greater than its pressor activity. This shows that modification of the structure of the vasopressins (replacement of the basic residue in position 8 by leucine) affects pressor potency more strongly than antidiuretic potency. Although this analogue still possesses oxytocin-like activity, it may nevertheless be regarded as an artificial antidiuretic hormone with low pressor activity.

The above findings seemed to show that the side-chain in position 3 of the oxytocin molecule can be changed from an aliphatic to an aromatic structure without drastically affecting the oxytotic activity. This conclusion, however, proved to be fallacious when we examined an analogue with tyrosine in position 3. This aromatic analogue, *Tyr³-oxytocin*, differing from *Phe³-oxytocin* only by an additional phenolic group, is almost devoid of biological activity. We also investigated the influence of another aromatic residue by introducing tryptophan in position 3. The oxytotic activities of this compound, *Try³-oxytocin*, are again negligible. Furthermore no pressor activity can be detected; indeed, this compound showed a certain depressor effect on the blood pressure of the rat and the spinal cat.

Thus, structural tolerance is much more restricted than might have been suspected from an examination of the first members of this series of analogues.

When the analogous modifications were effected at position 3 of lysine-vasopressin (Table III), similar

¹⁵ C. N. SMYTH, Brit. med. J. 1, 856 (1958).

¹⁶ B. BERDE and K. SAAMELI, Acta endocrin. 32, 391 (1959).

Oxytocin-like activities (in international units per mg)				Vasopressin-like activities (in international units per mg)			Occurs in the posterior pituitary of
rat uterus (isolated)	cat uterus (in situ)	chicken blood pressure	rabbit mammary gland	rat blood pressure	cat blood pressure	rat antidiuresis	
450 ± 30	450 ± 30	450 ± 30	450 ± 30	5 ± 1	4 ± 1	5 ± 1	vertebrates
5 ± 0.5	—	40 ± 5	60 ± 10	270 ± 20	306 ± 13	~250	pig, hippopotamus
~20	—	~60	~70	~400	~400	~400	most mammals
~75	—	~150	~100	~125	—	—	non-mammalian vertebrates

observations were made. Replacement of phenylalanine by tyrosine—*Tyr³-Lys⁸-vasopressin*—or tryptophane—*Try³-Lys⁸-vasopressin*—greatly attenuates biological activity. Introduction of serine in the same position—*Ser³-Lys⁸-vasopressin*—has the same result. On the other hand, introduction of isoleucine in this position gives a compound *Ileu³-Lys⁸-vasopressin* or *Lys⁸-oxytocin* or *lysine-vasotocin* which, like *Phe³-oxytocin* discussed above, is structurally and biologically intermediate between lysine-vasopressin and oxytocin. However, the pressor activity of lysine-vasotocin is more pronounced than its antidiuretic activity.

From these studies on systematic variations in position 3 of oxytocin and lysine-vasopressin, it can be concluded that only a limited number of modifications lead to compounds which retain any noteworthy biological properties.

Variations in Position 8. It is interesting to investigate the effect of modifying the molecule at position 8 (i.e. outside of the disulphide ring) for, in the natural neurohypophysial hormones, variations also occur in this position (Table I).

In order to study the influence of relatively small changes, we replaced the leucine of the oxytocin molecule by isoleucine and by valine (Table IV). It is hardly surprising that these small changes should have only a slight influence on biological activity. Nevertheless, it is noteworthy that *Ileu⁸-oxytocin* exerts an even stronger effect than oxytocin itself on the cat uterus *in situ*. This is the first synthetic analogue of oxytocin possessing a higher activity than the natural hormone.

The replacement of leucine by valine in position 8 of oxytocin gives *Val⁸-oxytocin* and corresponds to the removal of a —CH₂— group from one of the side-chains. This slight modification has almost no influence on the

activity as measured on the cat uterus *in situ*, but roughly halves the activity as determined on the isolated rat uterus.

By replacing leucine in oxytocin by the basic amino acid lysine, we obtained *Lys⁸-oxytocin* (*lysine-vasotocin*), which is a close analogue of *arginine-vasotocin* recently found in non-mammalian vertebrates. Both compounds have properties intermediate between those of oxytocin and those of the vasopressins. Hence introduction of a basic group in position 8 of oxytocin enhances the vasopressin-like qualities of the oxytocin molecule and attenuates its oxytocin-like properties.

Examples of variations in position 8 of the vasopressins are found in nature: *lysine-vasopressin* occurs in the pig and the hippopotamus, *arginine-vasopressin* in all other mammalian vertebrates investigated so far (Table I). Arginine-vasopressin is somewhat more potent than lysine-vasopressin in every respect. DU VIGNEAUD and KATSOYANNIS have introduced a histidine residue in position 8 thus obtaining *His⁸-vasopressin* (Table V). This modification greatly attenuates all the biological activities, the vasopressin-like qualities being particularly affected. Leucine was introduced in the same position by our group and by KATSOYANNIS of DU VIGNEAUD's group independently. This modification yields *oxypressin* (*Phe³-oxytocin* = *Leu⁸-vasopressin*), a compound discussed above, which has properties intermediate between those of oxytocin and the vasopressins.

It would appear from the data in Table V that replacement of the basic amino acid in position 8 of the vasopressins by a neutral amino acid greatly weakens the vasopressin-like activities without much affecting the oxytocin-like properties. It might therefore be expected that introduction of the slightly basic histidine

Tab. II. Variations in position 3 of oxytocin

Name and chemical formula	$R_2 = -\text{CH}_2-\text{C}_6\text{H}_4-\text{OH}$	$R_8 = -\text{CH}_2-\overset{\text{CH}_3}{\underset{ }{\text{CH}}}-\text{CH}_3$
Oxytocin	H-CyS-Tyr- <u>Ileu</u> -Glu(NH ₂)-Asp(NH ₂)-CyS-Pro-Leu-Gly-NH ₂	$R_3 = -\overset{\text{CH}_3}{\underset{ }{\text{CH}}}-\text{CH}_2-\text{CH}_3$
Val ³ -oxytocin	H-CyS-Tyr- <u>Val</u> -Glu(NH ₂)-Asp(NH ₂)-CyS-Pro-Leu-Gly-NH ₂	$R_3 = -\overset{\text{CH}_3}{\underset{ }{\text{CH}}}-\text{CH}_3$
Leu ³ -oxytocin	H-CyS-Tyr- <u>Leu</u> -Glu(NH ₂)-Asp(NH ₂)-CyS-Pro-Leu-Gly-NH ₂	$R_3 = -\text{CH}_2-\overset{\text{CH}_3}{\underset{ }{\text{CH}}}-\text{CH}_3$
Phe ³ -oxytocin = Oxypressin	H-CyS-Tyr- <u>Phe</u> -Glu(NH ₂)-Asp(NH ₂)-CyS-Pro-Leu-Gly-NH ₂	$R_3 = -\text{CH}_2-\text{C}_6\text{H}_5$
Tyr ³ -oxytocin	H-CyS-Tyr- <u>Tyr</u> -Glu(NH ₂)-Asp(NH ₂)-CyS-Pro-Leu-Gly-NH ₂	$R_3 = -\text{CH}_2-\text{C}_6\text{H}_4-\text{OH}$
Try ³ -oxytocin	H-CyS-Tyr- <u>Try</u> -Glu(NH ₂)-Asp(NH ₂)-CyS-Pro-Leu-Gly-NH ₂	$R_3 = -\text{CH}_2-\text{C}(\text{CH}=\text{CH}-\text{NH})-\text{C}_6\text{H}_4$

Tab. III. Variations in position 3 of lysine-vasopressin

Name and chemical formula	$R_2 = -\text{CH}_2-\text{C}_6\text{H}_4-\text{OH}$	$R_8 = -\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{NH}_2$
Lysine-vasopressin	H-CyS-Tyr- <u>Phe</u> -Glu(NH ₂)-Asp(NH ₂)-CyS-Pro-Lys-Gly-NH ₂	$R_3 = -\text{CH}_2-\text{C}_6\text{H}_5$
Tyr ³ -Lys ⁸ -vasopressin	H-CyS-Tyr- <u>Tyr</u> -Glu(NH ₂)-Asp(NH ₂)-CyS-Pro-Lys-Gly-NH ₂	$R_3 = -\text{CH}_2-\text{C}_6\text{H}_4-\text{OH}$
Try ³ -Lys ⁸ -vasopressin	H-CyS-Tyr- <u>Try</u> -Glu(NH ₂)-Asp(NH ₂)-CyS-Pro-Lys-Gly-NH ₂	$R_3 = -\text{CH}_2-\text{C}(\text{CH}=\text{CH}-\text{NH})-\text{C}_6\text{H}_4$
Ileu ³ -Lys ⁸ -vasopressin = Lysine-vasotocin	H-CyS-Tyr- <u>Ileu</u> -Glu(NH ₂)-Asp(NH ₂)-CyS-Pro-Lys-Gly-NH ₂	$R_3 = -\overset{\text{CH}_3}{\underset{ }{\text{CH}}}-\text{CH}_2-\text{CH}_3$
Ser ³ -Lys ⁸ -vasopressin	H-CyS-Tyr- <u>Ser</u> -Glu(NH ₂)-Asp(NH ₂)-CyS-Pro-Lys-Gly-NH ₂	$R_3 = -\text{CH}_2-\text{OH}$

Oxytocin-like activities (in international units per mg)				Vasopressin-like activities (in international units per mg)			Bibliography	
rat uterus (isolated)	cat uterus (<i>in situ</i>)	chicken blood pressure	rabbit mammary gland	rat blood pressure	cat blood pressure	rat antidiuresis	S synthesis P pharmacology	* contains also pharmacological data
450 ± 30	450 ± 30	450 ± 30	450 ± 30	5 ± 1	4 ± 1	5 ± 1	Occurs in nature	
59 ± 8	226 ± 17	58 ± 4	207 ± 14	~0.2	~0.5	~0.8	S BOISSONNAS, GUTTMANN, JAQUENOUD, WALLER, <i>Helv. chim. Acta</i> 39, 1421 (1956) P BERDE, DOEPFNER, KONZETT, <i>Brit. J. Pharmacol. Chemotherapy</i> 12, 209 (1957); BERDE, CERLETTI, KONZETT, Symposium on oxytocin, Montevideo (1959)	
45 ± 7	61 ± 0.7	42 ± 1	101 ± 13	~0.3	—	~0.2	S BOISSONNAS, GUTTMANN, JAQUENOUD, WALLER, <i>Helv. chim. Acta</i> 39, 1421 (1956) P BERDE, DOEPFNER, KONZETT, <i>Brit. J. Pharmacol. Chemotherapy</i> 12, 209 (1957); BERDE, STÜRMER (pers. comm.)	
~20	~25	~30	~60	~3	~5	~30	S BOISSONNAS, GUTTMANN, JAQUENOUD, WALLER, <i>Helv. chim. Acta</i> 39, 1421 (1956); KATSOYANNIS, <i>J. Amer. chem. Soc.</i> 79, 109 (1957)* P BERDE, DOEPFNER, KONZETT, <i>Brit. J. Pharmacol. Chemotherapy</i> 12, 209 (1957)	
0.10 ± 0.03	—	~0.03	1.5 ± 0.3	~0.01	~0.01	—	S BOISSONNAS, GUTTMANN, <i>Helv. chim. Acta</i> 43, 190 (1960)* P BERDE, CERLETTI, KONZETT, Symposium on oxytocin, Montevideo (1959)	
0.04 ± 0.01	—	~0.1	0.10 ± 0.06	< 0.01	< 0.01	—	S GUTTMANN, BOISSONNAS, <i>Helv. chim. Acta</i> 43, 200 (1960)* P BERDE, CERLETTI, KONZETT, Symposium on oxytocin, Montevideo (1959)	

Oxytocin-like activities (in international units per mg)				Vasopressin-like activities (in international units per mg)			Bibliography	
rat uterus (isolated)	cat uterus (<i>in situ</i>)	chicken blood pressure	rabbit mammary gland	rat blood pressure	cat blood pressure	rat antidiuresis	S synthesis P pharmacology	* contains also pharmacological data
5 ± 0.5	—	40 ± 5	60 ± 10	270 ± 20	306 ± 13	~250	Occurs in nature	
~0.01	—	~0.1	~0.2	1.6 ± 0.2	2.6 ± 0.7	0.18 ± 0.08	S BOISSONNAS, GUTTMANN, <i>Helv. chim. Acta</i> 43, 190 (1960)* P BERDE, CERLETTI, KONZETT, Symposium on oxytocin, Montevideo (1959)	
< 0.01	—	~0.08	< 0.01	~0.07	~0.3	—	S GUTTMANN, BOISSONNAS, <i>Helv. chim. Acta</i> 43, 200 (1960)* P BERDE, CERLETTI, KONZETT, Symposium on oxytocin, Montevideo (1959)	
78 ± 10	—	210 ± 3	180 ± 25	130 ± 13	—	24 ± 3	S BOISSONNAS, HUGUENIN, <i>Helv. chim. Acta</i> 43, 182 (1960)*, and pers. comm.; KIMBROUGH, DU VIGNEAUD, <i>J. biol. Chem.</i> 236, 778 (1961)* P BERDE, CERLETTI, KONZETT, Symposium on oxytocin, Montevideo (1959); BERDE, STÜRMER (pers. comm.)	
< 0.01	~0.04	< 0.01	~0.04	< 0.01	—	~0.02	S GUTTMANN, BOISSONNAS, <i>Helv. chim. Acta</i> 43, 200 (1960)* P BERDE, CERLETTI, KONZETT, Symposium on oxytocin, Montevideo (1959)	

Tab. IV. Variations in position 8 of oxytocin

Name and chemical formula		$R_2 = -\text{CH}_2-\text{C}_6\text{H}_4-\text{OH}$	$R_3 = -\text{CH}(\text{CH}_3)-\text{CH}_2-\text{CH}_3$
Oxytocin	$\text{H-CyS-Tyr-Ileu-Glu(NH}_2\text{)-Asp(NH}_2\text{)-CyS-Pro-}\underline{\text{Leu}}\text{-Gly-NH}_2$		$R_8 = -\text{CH}_2-\text{CH}(\text{CH}_3)-\text{CH}_3$
Ileu ⁸ -oxytocin	$\text{H-CyS-Tyr-Ileu-Glu(NH}_2\text{)-Asp(NH}_2\text{)-CyS-Pro-}\underline{\text{Ileu}}\text{-Gly-NH}_2$		$R_8 = -\text{CH}(\text{CH}_3)-\text{CH}_2-\text{CH}_3$
Val ⁸ -oxytocin	$\text{H-CyS-Tyr-Ileu-Glu(NH}_2\text{)-Asp(NH}_2\text{)-CyS-Pro-}\underline{\text{Val}}\text{-Gly-NH}_2$		$R_8 = -\text{CH}(\text{CH}_3)-\text{CH}_3$
Lys ⁸ -oxytocin = Lysine-vasotocin	$\text{H-CyS-Tyr-Ileu-Glu(NH}_2\text{)-Asp(NH}_2\text{)-CyS-Pro-}\underline{\text{Lys}}\text{-Gly-NH}_2$		$R_8 = -\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{NH}_2$
Arg ⁸ -oxytocin = Arginine-vasotocin	$\text{H-CyS-Tyr-Ileu-Glu(NH}_2\text{)-Asp(NH}_2\text{)-CyS-Pro-}\underline{\text{Arg}}\text{-Gly-NH}_2$		$R_8 = -\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{NH}-\underset{\text{NH}}{\underset{\parallel}{\text{C}}}-\text{NH}_2$

Tab. V. Variations in position 8 of lysine-vasopressin

Name and chemical formula		$R_2 = -\text{CH}_2-\text{C}_6\text{H}_4-\text{OH}$	$R_3 = -\text{CH}_2-\text{C}_6\text{H}_5$
Lysine-vasopressin	$\text{H-CyS-Tyr-Phe-Glu(NH}_2\text{)-Asp(NH}_2\text{)-CyS-Pro-}\underline{\text{Lys}}\text{-Gly-NH}_2$		$R_8 = -\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{NH}_2$
Arginine-vasopressin	$\text{H-CyS-Tyr-Phe-Glu(NH}_2\text{)-Asp(NH}_2\text{)-CyS-Pro-}\underline{\text{Arg}}\text{-Gly-NH}_2$		$R_8 = -\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{NH}-\underset{\text{NH}}{\underset{\parallel}{\text{C}}}-\text{NH}_2$
His ⁸ -vasopressin	$\text{H-CyS-Tyr-Phe-Glu(NH}_2\text{)-Asp(NH}_2\text{)-CyS-Pro-}\underline{\text{His}}\text{-Gly-NH}_2$		$R_8 = -\text{CH}_2-\underset{\text{CH-N}}{\underset{\parallel}{\text{C}}}-\text{NH}$
Phe ⁸ -oxytocin = Oxypressin = Leu ⁸ -vasopressin	$\text{H-CyS-Tyr-Phe-Glu(NH}_2\text{)-Asp(NH}_2\text{)-CyS-Pro-}\underline{\text{Leu}}\text{-Gly-NH}_2$		$R_8 = -\text{CH}_2-\text{CH}(\text{CH}_3)-\text{CH}_3$

in this position would furnish a compound with properties intermediate between those of the vasopressins and those of Leu⁸-vasopressin. However, as we have seen above, this modification decreases all activities to a level much below that which might have been expected. This example serves to emphasise how mis-

leading it is to generalise from a study of a limited number of analogues.

Variations in Position 2. The replacement of tyrosine in oxytocin by phenylalanine, i.e. the suppression of the phenolic group, was accomplished independently by our group and by DU VIGNEAUD's team yielding *Phe*²-

Oxytocin-like activities (in international units per mg)				Vasopressin-like activities (in international units per mg)			Bibliography	
rat uterus (isolated)	cat uterus (<i>in situ</i>)	chicken blood pressure	rabbit mammary gland	rat blood pressure	cat blood pressure	rat antidiuresis	S synthesis P pharmacology	* contains also pharmacological data
450 ± 30	450 ± 30	450 ± 30	450 ± 30	5 ± 1	4 ± 1	5 ± 1	Occurs in nature	
289 ± 21	563 ± 74	498 ± 37	328 ± 21	6.3 ± 0.8	—	1.1 ± 0.1	S JAQUENOUD, BOISSONNAS, <i>Helv. chim. Acta</i> 44, 113 (1961)* P BERDE, KONZETT, <i>Medicina exp.</i> 2, 317 (1960)	
200 ± 15	380 ± 40	280 ± 17	310 ± 20	9 ± 1	—	0.8 ± 0.1	S JAQUENOUD, BOISSONNAS, <i>Helv. chim. Acta</i> 44, 113 (1961)* P KONZETT, BERDE, STÜRMER (pers. comm.)	
78 ± 10	—	210 ± 3	180 ± 25	130 ± 13	—	24 ± 3	S BOISSONNAS, HUGUENIN, <i>Helv. chim. Acta</i> 43, 182 (1960)* and pers. comm.; KIMBROUGH, DU VIGNEAUD, <i>J. biol. Chem.</i> 236, 778 (1961)* P BERDE, CERLETTI, KONZETT, <i>Symposium on oxytocin, Montevideo</i> (1959); BERDE, STÜRMER (pers. comm.)	
~75	—	~150	~100	~125	—	—	Occurs in nature	

Oxytocin-like activities (in international units per mg)				Vasopressin-like activities (in international units per mg)			Bibliography	
rat uterus (isolated)	cat uterus (<i>in situ</i>)	chicken blood pressure	rabbit mammary gland	rat blood pressure	cat blood pressure	rat antidiuresis	S synthesis P pharmacology	* contains also pharmacological data
5 ± 0.5	—	40 ± 5	60 ± 10	270 ± 20	306 ± 13	~250	Occurs in nature	
~20	—	~60	~70	~400	~400	~400	Occurs in nature	
~1.5	—	~4.6	—	~1.5	—	—	S KATSOYANNIS, DU VIGNEAUD, <i>Arch. Biochem. Biophys.</i> 78, 555 (1958)*	
~20	~25	~30	~60	~3	~5	~30	S BOISSONNAS, GUTTMANN, JAQUENOUD, WALLER, <i>Helv. chim. Acta</i> 39, 1421 (1956); KATSOYANNIS, <i>J. Amer. chem. Soc.</i> 79, 109 (1957)* P BERDE, DOEPFNER, KONZETT, <i>Brit. J. Pharmacol. Chemotherapy</i> 12, 209 (1957).	

oxytocin. It can be seen from the data in Table VI that some types of activities are more affected than others by this variation. Nevertheless, appreciable oxytocin-like effects are still in evidence. This illustrates that the hydroxyl group is not vital for the oxytocic activity. The effect of methylating the phenolic group of tyrosine

was investigated by DU VIGNEAUD's group. They found that this analogue—(*O-Me*)*Tyr*²-*oxytocin*—has an unexpectedly low activity, indeed, that it has a certain antagonistic action on vasopressin. Methylation of the amino-group of the tyrosine in position 2, which is linked to the half-cystine residue occupying position

Tab. VI. Variations in position 2 of oxytocin

Name and chemical formula	$R_3 = \begin{array}{c} \text{CH}_3 \\ \\ -\text{CH}-\text{CH}_2-\text{CH}_3 \end{array}$	$R_8 = \begin{array}{c} \text{CH}_3 \\ \\ -\text{CH}_2-\text{CH}-\text{CH}_3 \end{array}$
Oxytocin	H-CyS- <u>Tyr-Ileu-Glu(NH₂)-Asp(NH₂)</u> -CyS-Pro-Leu-Gly-NH ₂	$R_2 = -\text{CH}_2-\text{C}_6\text{H}_4-\text{OH}$
Phe ² -oxytocin (= Desoxy-oxytocin)	H-CyS- <u>Phe-Ileu-Glu(NH₂)-Asp(NH₂)</u> -CyS-Pro-Leu-Gly-NH ₂	$R_2 = -\text{CH}_2-\text{C}_6\text{H}_5$
(N-Me)Tyr ² -oxytocin	H-CyS- <u>(N-Me)Tyr-Ileu-Glu(NH₂)-Asp(NH₂)</u> -CyS-Pro-Leu-Gly-NH ₂ (-CH ₃ instead of -H on the nitrogen of tyrosine)	$R_2 = -\text{CH}_2-\text{C}_6\text{H}_4-\text{OH}$
(O-Me)Tyr ² -oxytocin = = (<i>p</i> -methoxy) Phe ² -oxytocin	H-CyS- <u>(O-Me)Tyr-Ileu-Glu(NH₂)-Asp(NH₂)</u> -CyS-Pro-Leu-Gly-NH ₂	$R_2 = -\text{CH}_2-\text{C}_6\text{H}_4-\text{OCH}_3$
Ser ² -oxytocin	H-CyS- <u>Ser-Ileu-Glu(NH₂)-Asp(NH₂)</u> -CyS-Pro-Leu-Gly-NH ₂	$R_2 = -\text{CH}_2-\text{OH}$

Tab. VII. Variations in position 2 of lysine-vasopressin

Name and chemical formula	$R_3 = -\text{CH}_2-\text{C}_6\text{H}_5$	$R_8 = -\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{NH}_2$
Lysine-vasopressin	H-CyS- <u>Tyr-Phe-Glu(NH₂)-Asp(NH₂)</u> -CyS-Pro-Lys-Gly-NH ₂	$R_2 = -\text{CH}_2-\text{C}_6\text{H}_4-\text{OH}$
Phe ² -Lys ⁸ -vasopressin	H-CyS- <u>Phe-Phe-Glu(NH₂)-Asp(NH₂)</u> -CyS-Pro-Lys-Gly-NH ₂	$R_2 = -\text{CH}_2-\text{C}_6\text{H}_5$
His ² -Lys ⁸ -vasopressin	H-CyS- <u>His-Phe-Glu(NH₂)-Asp(NH₂)</u> -CyS-Pro-Lys-Gly-NH ₂	$R_2 = -\text{CH}_2-\text{C}(\text{NH})=\text{CH}$

1, was accomplished by our group. This modification also gives a compound—(*N*-Me)Tyr²-oxytocin—with an unexpectedly low activity.

To investigate the effect of substituting an alcoholic hydroxyl group for a phenolic hydroxyl group, we replaced tyrosine by serine. The compound obtained—Ser²-oxytocin—is almost devoid of biological activity.

Replacement of the tyrosine in position 2 of lysine-vasopressin (Table VII) by phenylalanine yielded a compound—Phe²-Lys⁸-vasopressin—with interesting properties. The inherent oxytocin-like qualities of lysine-vasopressin are reduced to a very low level by this modification, but the pressor activity is still considerable, viz. one-fifth of its original value, whereas

Oxytocin-like activities (in international units per mg)				Vasopressin-like activities (in international units per mg)			Bibliography	
rat uterus (isolated)	cat uterus (<i>in situ</i>)	chicken blood pressure	rabbit mammary gland	rat blood pressure	cat blood pressure	rat antidiuresis	S synthesis P pharmacology	* contains also pharmacological data
450 ± 30	450 ± 30	450 ± 30	450 ± 30	5 ± 1	4 ± 1	5 ± 1	Occurs in nature	
32 ± 2	168 ± 12	63 ± 9	141 ± 21	~ 0.4	~ 3	~ 0.5	S JAQUENOUD, BOISSONNAS, <i>Helv. chim. Acta</i> 42, 788 (1959)*; BODANSZKY, DU VIGNEAUD, <i>J. Amer. chem. Soc.</i> 81, 1258, 6072 (1959)* P KONZETT, BERDE, <i>Brit. J. Pharmacol. Chemotherapy</i> 14, 333 (1959); BERDE, KONZETT (pers. comm.)	
1.2 ± 0.4	—	0.32 ± 0.05	3.1 ± 0.7	—	—	< 0.01	S HUGUENIN, BOISSONNAS, <i>Helv. chim. Acta</i> 44, 213 (1961)* P KONZETT, BERDE, STÜRMER (pers. comm.)	
~ 5	—	~ 5	—	Anti (3000:1)	—	—	S LAW, DU VIGNEAUD, <i>J. Amer. chem. Soc.</i> 82, 4579 (1960)*	
< 0.01	—	< 0.01	< 0.01	< 0.01	—	< 0.01	S GUTTMANN, BOISSONNAS, <i>Helv. chim. Acta</i> 43, 200 (1960)* P BERDE, CERLETTI, KONZETT, <i>Symposium on oxytocin, Montevideo</i> (1959)	

Oxytocin-like activities (in international units per mg)				Vasopressin-like activities (in international units per mg)			Bibliography	
rat uterus (isolated)	cat uterus (<i>in situ</i>)	chicken blood pressure	rabbit mammary gland	rat blood pressure	cat blood pressure	rat antidiuresis	S synthesis P pharmacology	* contains also pharmacological data
5 ± 0.5	—	40 ± 5	60 ± 10	270 ± 20	306 ± 13	~ 250	Occurs in nature	
~ 0.3	—	~ 0.15	~ 2.5	55 ± 7	—	20 ± 2	S BOISSONNAS, GUTTMANN, <i>Helv. chim. Acta</i> 43, 190 (1960)* P BERDE, CERLETTI, KONZETT, <i>Symposium on oxytocin, Montevideo</i> (1959)	
< 0.01	—	< 0.01	< 0.01	< 0.01	~ 0.08	—	S GUTTMANN, BOISSONNAS, <i>Helv. chim. Acta</i> 43, 200 (1960)* P BERDE, CERLETTI, KONZETT, <i>Symposium on oxytocin, Montevideo</i> (1959)	

the antidiuretic potency is one-twelfth of its former value. This analogue is therefore much more selectively pressor than vasopressin. This has been confirmed in clinical experiments¹⁷.

Introduction of histidine in position 2 gave *His*²-*Lys*⁸-*vasopressin*. As might be expected, this modification annihilates all biological activity.

Simultaneous variations in positions 2 and 3. From the data in Tables VIII and IX, it is evident that simultaneous modifications in positions 2 and 3 generally lead to complete loss of biological activity. There are, however, two exceptions: *Phe*²-*Phe*³-*oxytocin* (= *desoxy-*

¹⁷ U. GUHL, *Schweiz. med. Wschr.* 91, 798 (1961).

Tab. VIII. Variations in positions 2 and 3 of oxytocin

Name and chemical formula		$R_8 = -\text{CH}_2-\overset{\text{CH}_3}{\underset{ }{\text{CH}}}-\text{CH}_3$	
Oxytocin	$\text{H-CyS-Tyr-Ileu-Glu(NH}_2\text{)-Asp(NH}_2\text{)-CyS-Pro-Leu-Gly-NH}_2$	$R_2 = -\text{CH}_2-\text{C}_6\text{H}_4\text{-OH}$	$R_3 = -\text{CH}_2-\overset{\text{CH}_3}{\underset{ }{\text{CH}}}-\text{CH}_2-\text{CH}_3$
Phe ² -Tyr ³ -oxytocin	$\text{H-CyS-Phe-Tyr-Glu(NH}_2\text{)-Asp(NH}_2\text{)-CyS-Pro-Leu-Gly-NH}_2$	$R_2 = -\text{CH}_2-\text{C}_6\text{H}_5$	$R_3 = -\text{CH}_2-\text{C}_6\text{H}_4\text{-OH}$
Phe ² -Phe ³ -oxytocin	$\text{H-CyS-Phe-Phe-Glu(NH}_2\text{)-Asp(NH}_2\text{)-CyS-Pro-Leu-Gly-NH}_2$		$R_2 = R_3 = -\text{CH}_2-\text{C}_6\text{H}_5$
Ser ² -His ³ -oxytocin	$\text{H-CyS-Ser-His-Glu(NH}_2\text{)-Asp(NH}_2\text{)-CyS-Pro-Leu-Gly-NH}_2$	$R_2 = -\text{CH}_2\text{-OH}$	$R_3 = -\text{CH}_2-\text{C}(\text{NH})\text{CH}(\text{CH}=\text{N})\text{CH}$
His ² -Phe ³ -oxytocin	$\text{H-CyS-His-Phe-Glu(NH}_2\text{)-Asp(NH}_2\text{)-CyS-Pro-Leu-Gly-NH}_2$	$R_2 = -\text{CH}_2-\text{C}(\text{NH})\text{CH}(\text{CH}=\text{N})\text{CH}$	$R_3 = -\text{CH}_2-\text{C}_6\text{H}_5$

Tab. IX. Variations in positions 2 and 3 of lysine-vasopressin

Name and chemical formula		$R_3 = -\text{CH}_2\text{-CH}_2\text{-CH}_2\text{-CH}_2\text{-NH}_2$	
$\text{H-CyS-Tyr-Phe-Glu(NH}_2\text{)-Asp(NH}_2\text{)-CyS-Pro-Lys-Gly-NH}_2$		$R_2 = -\text{CH}_2-\text{C}_6\text{H}_4\text{-OH}$	$R_3 = -\text{CH}_2-\text{C}_6\text{H}_5$
Lysine-vasopressin			
$\text{H-CyS-Phe-Tyr-Glu(NH}_2\text{)-Asp(NH}_2\text{)-CyS-Pro-Lys-Gly-NH}_2$		$R_2 = -\text{CH}_2-\text{C}_6\text{H}_5$	$R_3 = -\text{CH}_2-\text{C}_6\text{H}_4\text{-OH}$
Phe ² -Tyr ³ -Lys ⁸ -vasopressin			
$\text{H-CyS-Phe-Ileu-Glu(NH}_2\text{)-Asp(NH}_2\text{)-CyS-Pro-Lys-Gly-NH}_2$		$R_2 = -\text{CH}_2-\text{C}_6\text{H}_5$	$R_3 = -\text{CH}_2-\overset{\text{CH}_3}{\underset{ }{\text{CH}}}-\text{CH}_2-\text{CH}_3$
Phe ² -Ileu ³ -Lys ⁸ -vasopressin = Desoxy-lysine-vasotocin			
$\text{H-CyS-Ser-Ileu-Glu(NH}_2\text{)-Asp(NH}_2\text{)-CyS-Pro-Lys-Gly-NH}_2$		$R_2 = -\text{CH}_2\text{-OH}$	$R_3 = -\text{CH}_2-\overset{\text{CH}_3}{\underset{ }{\text{CH}}}-\text{CH}_2-\text{CH}_3$
Ser ² -Ileu ³ -Lys ⁸ -vasopressin			
$\text{H-CyS-Ser-His-Glu(NH}_2\text{)-Asp(NH}_2\text{)-CyS-Pro-Lys-Gly-NH}_2$		$R_2 = -\text{CH}_2\text{-OH}$	$R_3 = -\text{CH}_2-\text{C}(\text{NH})\text{CH}(\text{CH}=\text{N})\text{CH}$
Ser ² -His ³ -Lys ⁸ -vasopressin			
$\text{H-CyS-His-Ser-Glu(NH}_2\text{)-Asp(NH}_2\text{)-CyS-Pro-Lys-Gly-NH}_2$		$R_2 = -\text{CH}_2-\text{C}(\text{NH})\text{CH}(\text{CH}=\text{N})\text{CH}$	$R_3 = -\text{CH}_2\text{-OH}$
His ² -Ser ³ -Lys ⁸ -vasopressin			

Oxytocin-like activities (in international units per mg)				Vasopressin-like activities (in international units per mg)			Bibliography	
rat uterus (isolated)	cat uterus (<i>in situ</i>)	chicken blood pressure	rabbit mammary gland	rat blood pressure	cat blood pressure	rat antidiuresis	S synthesis P pharmacology	* contains also pharmacological data
450 ± 30	450 ± 30	450 ± 30	450 ± 30	5 ± 1	4 ± 1	5 ± 1	Occurs in nature	
< 0.01	—	< 0.01	—	< 0.01	—	~ 0.001	S BOISSONNAS, GUTTMANN, <i>Helv. chim. Acta</i> 43, 190 (1960)* P KONZETT, BERDE (pers. comm.)	
3.3 ± 0.3	—	5.4 ± 0.9	24.6 ± 9.0	~ 0.9	—	5.7 ± 0.6	S BOISSONNAS, GUTTMANN, <i>Helv. chim. Acta</i> 43, 190 (1960)* P BERDE, CERLETTI, KONZETT, Symposium on oxytocin, Montevideo (1959)	
< 0.01	—	~ 0.03	—	—	< 0.01	—	S GUTTMANN, BOISSONNAS, <i>Helv. chim. Acta</i> 43, 200 (1960)* P BERDE, CERLETTI, KONZETT, Symposium on oxytocin, Montevideo (1959)	
< 0.01	—	< 0.01	—	< 0.01	< 0.01	—	S GUTTMANN, BOISSONNAS, <i>Helv. chim. Acta</i> 43, 200 (1960)* P BERDE, CERLETTI, KONZETT, Symposium on oxytocin, Montevideo (1959)	

Oxytocin-like activities (in international units per mg)				Vasopressin-like activities (in international units per mg)			Bibliography	
rat uterus (isolated)	cat uterus (<i>in situ</i>)	chicken blood pressure	rabbit mammary gland	rat blood pressure	cat blood pressure	rat antidiuresis	S synthesis P pharmacology	* contains also pharmacological data
5 ± 0.5	—	40 ± 5	60 ± 10	270 ± 20	306 ± 13	~ 250	Occurs in nature	
< 0.01	—	< 0.01	—	0.14 ± 0.01	—	0.013 ± 0.002	S BOISSONNAS, GUTTMANN, <i>Helv. chim. Acta</i> 43, 190 (1960)* P KONZETT, BERDE (pers. comm.)	
1.0 ± 0.1	—	~ 8	12 ± 3	32 ± 6	—	1.0 ± 0.1	S JAQUENOUD, BOISSONNAS (to be published) P BERDE, KONZETT, STÜRMER (pers. comm.)	
< 0.01	—	< 0.01	~ 0.01	~ 0.1	—	~ 0.06	S GUTTMANN, BOISSONNAS, <i>Helv. chim. Acta</i> 43, 200 (1960)* P BERDE, CERLETTI, KONZETT, Symposium on oxytocin, Montevideo (1959)	
< 0.01	—	< 0.02	—	< 0.02	~ 0.04	—	S GUTTMANN, BOISSONNAS, <i>Helv. chim. Acta</i> 43, 200 (1960)* P BERDE, CERLETTI, KONZETT, Symposium on oxytocin, Montevideo (1959)	
< 0.01	—	—	< 0.01	—	< 0.01	—	S GUTTMANN, BOISSONNAS, <i>Helv. chim. Acta</i> 43, 200 (1960)* P BERDE, CERLETTI, KONZETT, Symposium on oxytocin, Montevideo (1959)	

oxypressin) and *Phe*²-*Ileu*³-*Lys*⁸-*vasopressin* (= *desoxy-lysine-vasotocin*). Here again the removal of the phenolic hydroxyl of the two analogous compounds, oxypressin and lysine-vasotocin, described above, yields new substances having a lower, but still considerable activity which is rather selective. It can therefore be concluded that the removal of the hydroxyl group of the tyrosine residue lowers the biological activity but increases the selectivity of the compounds. The same is true of desoxy-oxytocin (= *Phe*²-oxytocin) and desoxy-lysine-vasopressin (= *Phe*²-lysine-vasopressin), as was shown above.

Another interesting observation in this series is that, merely by interchanging tyrosine and phenylalanine in lysine-vasopressin, a compound (*Phe*²-*Tyr*³-*Lys*⁸-*vasopressin*) is obtained which is almost devoid of biological activity.

Shortening of the peptide chain of oxytocin. Some years ago, a hexapeptide amide, representing the amidified ring of the oxytocin molecule deprived of its tripeptidic side-chain (Table X), was prepared by RESSLER in DU VIGNEAUD's laboratory. This lower homologue of oxytocin—*Des*-(*Pro*⁷-*Leu*⁸-*Gly*⁹)-*oxytocin*—retains about 1/150 of the activity of the natural hormone on the rat uterus and 1/400 on the rabbit mammary gland.

We have recently synthesized two other lower homologues of oxytocin from which the proline of position 7 or the glycine of position 9 was omitted. These two lower homologues (*Des*-*Pro*⁷-*oxytocin* and *Des*-*Gly*⁹-*oxytocin*) exhibit a residual activity similar to that of the cyclic hexapeptide mentioned above.

It is noteworthy that none of these three compounds affects the chicken blood pressure. In fact the two compounds with shortened side-chains actually inhibit the response to a subsequent dose of oxytocin, albeit only slightly. Shortening the side-chain of oxytocin seems to have less effect on the typical oxytocic effects on the uterus and the mammary gland than on the avian depressor activity.

Recently, in a preliminary communication, DU VIGNEAUD's group¹⁸ announced that the omission of the amino group of the first half-cystine residue of the oxytocin molecule yields an analogue (des-amino-oxytocin) which possesses a very high avian depressor action and an appreciable effect on the rat uterus and on the mammary gland. The absence of the N-terminal amino group of the oxytocin molecule would thus appear to have no detrimental influence on the biological activities of the molecule. In our opinion, it cannot be excluded that this modification may perhaps increase the resistance of the molecule to oxytocin-inactivating enzymes.

Lengthening of the peptide chain of oxytocin. As the above-mentioned results show, the terminal amino group of oxytocin does not seem to be necessary for

Tab. X. Shortening of the peptide chain of oxytocin

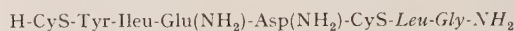
Name and chemical formula



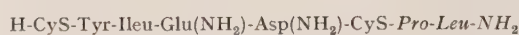
Oxytocin



Des-(*Pro*⁷-*Leu*⁸-*Gly*⁹)-oxytocin



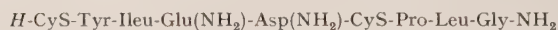
Des-*Pro*⁷-oxytocin



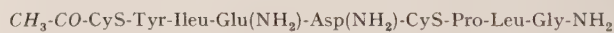
Des-*Gly*⁹-oxytocin

Tab. XI. Lengthening of the peptide chain of oxytocin

Name and chemical formula



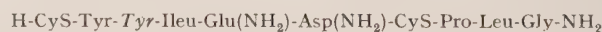
Oxytocin



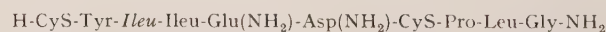
N-Acetyl-oxytocin



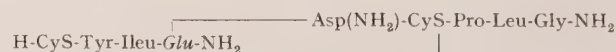
N-Glycyl-oxytocin



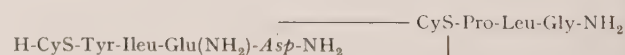
Homo-*Tyr*^{2,3}-oxytocin



Homo-*Ileu*^{2,3}-oxytocin



Isoglutamine⁴-oxytocin



Isoasparagine⁵-oxytocin

¹⁸ V. DU VIGNEAUD, G. WINESTOCK, V. V. S. MURTI, D. B. HOPE, R. D. KIMBROUGH, J. biol. Chem. 235, PC 64 (1960).

Oxytocin-like activities (in international units per mg)				Vasopressin-like activities (in international units per mg)			Bibliography	
rat uterus (isolated)	cat uterus (<i>in situ</i>)	chicken blood pressure	rabbit mammary gland	rat blood pressure	cat blood pressure	rat antidiuresis	S synthesis P pharmacology	* contains also pharmacological data
450 ± 30	450 ± 30	450 ± 30	450 ± 30	5 ± 1	4 ± 1	5 ± 1	Occurs in nature	
~ 3.3	—	< 0.03	~ 1.1	< 0.01	—	—	S RESSLER, Proc. Soc. exp. Biol. Med. 92, 725 (1956)*	
3.8 ± 0.9	~ 0.4	Anti (1000:1)	1.6 ± 0.2	~ 0.01	—	~ 0.01	S JAQUENOUD, BOISSONNAS, Helv. chim. Acta (in preparation)* P BERDE, KONZETT, STÜRMER (pers. comm.)	
4.2 ± 0.7	—	Anti (10000:1)	~ 3	Anti (10000:1)	—	~ 0.02	S JAQUENOUD, BOISSONNAS, Helv. chim. Acta (in preparation)* P BERDE, KONZETT, STÜRMER (pers. comm.)	

Oxytocin-like activities (in international units per mg)				Vasopressin-like activities (in international units per mg)			Bibliography	
rat uterus (isolated)	cat uterus (<i>in situ</i>)	chicken blood pressure	rabbit mammary gland	rat blood pressure	cat blood pressure	rat antidiuresis	S synthesis P pharmacology	* contains also pharmacological data
450 ± 30	450 ± 30	450 ± 30	450 ± 30	5 ± 1	4 ± 1	5 ± 1	Occurs in nature	
1.7 ± 0.2	—	Anti (5000:1)	2.1 ± 0.2	—	—	—	S BOISSONNAS, PECHÈRE, GUTTMANN (pers. comm.) P KONZETT, BERDE (pers. comm.)	
~ 1	—	~ 1 Anti (20:1)	—	~ 0.05	—	—	S DU VIGNEAUD, FITT, BODANSZKY, O'CONNELL, Proc. Soc. exp. Biol. Med. 104, 653 (1960)*	
Anti (500:1)	—	Anti (500:1)	~ 0.5	—	—	—	S GUTTMANN, JAQUENOUD, BOISSONNAS, KONZETT, BERDE, Naturwiss. 44, 632 (1957)* P KONZETT, Helv. Physiol. Acta 15, 419 (1957)	
~ 0.1	—	~ 0.05	~ 0.2	—	< 0.01	< 0.01	S JAQUENOUD, BOISSONNAS (pers. comm.) P BERDE, CERLETTI, KONZETT, Symposium on oxytocin, Montevideo (1959)	
< 0.01	—	< 0.05	—	Anti (500:1)	—	—	S RESSLER, DU VIGNEAUD, J. Amer. chem. Soc. 79, 4511 (1957)* P RESSLER, RACHELE, Proc. Soc. exp. Biol. Med. 98, 170 (1958)	
< 0.01	—	< 0.05	—	< 0.01	—	—	S LUTZ, RESSLER, NETTLETON, DU VIGNEAUD, J. Amer. chem. Soc. 81, 167 (1959)*	

biological activity; it is interesting to consider the consequences of acetylating this amino group. We prepared *N*-acetyl-oxytocin (Table XI) and found it to be only about 1/250 as active as oxytocin on the rat uterus and on the rabbit mammary gland. Furthermore, instead of the high avian depressor activity exhibited by the above-mentioned des-amino-oxytocin, *N*-acetyl-oxytocin shows a slight antagonist effect to oxytocin in the same test. These apparently contradictory results can nevertheless be reconciled if we assume that steric effects play a preponderant rôle in the mechanism of action of oxytocin and that any additional group is detrimental to biological activity.

DU VIGNEAUD's group recently prepared *N*-glycyl-oxytocin, which differs from our *N*-acetyl-oxytocin only by possessing an additional terminal amino group. This compound also possesses a low oxytocic activity on the rat uterus. It has low avian depressor potency and markedly antagonises simultaneous or subsequent doses of oxytocin in the same test.

Some years ago, our group prepared a homologue of oxytocin containing two tyrosine residues instead of one (*Homo-Tyr^{2,3}-oxytocin*). This homologue displayed slight but definite antagonism to oxytocin on the rat uterus as well as on the chicken blood pressure. This was the first observation of an oxytocin-antagonistic effect by an oxytocin-like compound and prompted us to synthesize another homologue of oxytocin containing two isoleucine residues instead of one (*Homo-Ileu^{2,3}-oxytocin*). However, this compound has hardly any oxytocic or anti-oxytocic activity.

Investigating the influence of the relative positions of the amide group and of the peptide chain linked to the carboxyl group of the glutamic or aspartic residues

of the oxytocin molecule, DU VIGNEAUD's team prepared *isoglutamine⁴-oxytocin* and *isoasparagine⁵-oxytocin*. Both are devoid of oxytocic activity. The first compound was also shown to be slightly antagonistic to the pressor effect of vasopressin.

Conclusions. This review shows that relationships which have so far emerged from a study of the chemical structures and the biological activities of the posterior pituitary hormones and their synthetic analogues are in most cases limited in application. When the first members of a series of analogues are investigated, it frequently seems to be easy to correlate biological properties and chemical peculiarities. However, when a greater number of compounds are investigated, the picture often becomes more complex. Furthermore, it is misleading to draw conclusions from only one type of biological assay, since different tests may give rather divergent quantitative results.

Valid conclusions can only be obtained by systematically comparing a fairly large number of compounds of the same chemical family in a battery of biological tests—as has been done in the present review.

Résumé. Afin de mettre en évidence l'influence de faibles modifications de structure sur les propriétés biologiques, les auteurs comparent, à l'aide d'une série de tableaux systématiques, les structures chimiques et les propriétés pharmacologiques des hormones post-hypophysaires avec celles de près de quarante de leurs analogues synthétiques, qui ont été étudiés d'une manière approfondie. Cette confrontation montre qu'il est possible de découvrir quelques relations limitées entre la spécificité biologique de ces corps et certaines particularités de leur structure.

Brèves communications – Kurze Mitteilungen – Brevi comunicazioni – Brief Reports

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The Effect of Sea Water on Cytochrome Oxidase and Oxidative Phosphorylation

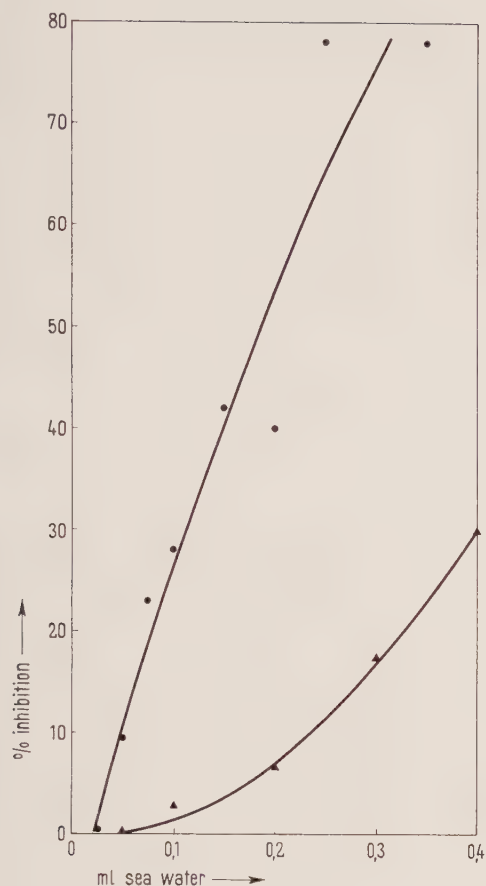
In the course of our work on the inhibition of cytochrome oxidase caused by a low molecular substance in the unfertilized sea urchin egg¹, it appeared important to check the influence that sea water might have on the oxidase activity. In fact, unless the eggs are thoroughly washed with some artificial solution, which did not seem to us to be advisable, it is practically impossible to avoid a contamination with sea water. As is shown in the diagram, 0.2 cm³ of sea water added to the reaction mixture (3.0 cm³ total) in the Warburg flask cause a 7% decrease of the oxygen consumption which reaches 30% when 0.4 cm³ of sea water are added. On the other hand, the influence on oxidative phosphorylation is very strong in-

deed. In fact, as is shown in the Figure, a 30% decrease of the P/O ratio is caused by as little as 0.1 cm³ of sea water and 0.3 cm³ depress the ratio by 80%. A series of experiments with artificial sea water from which individual ions were removed and then with each ion separately have proved that Ca⁺⁺ is responsible for the uncoupling effect. This is not surprising in view of the well known effect of Ca⁺⁺ as an uncoupler of oxidative phosphorylation².

The possibility that the effect of sea water might have been an apparent one, i.e. due to an inhibition of hexo-

¹ R. MAGGIO and A. MONROY, *Nature* 184, 68 (1959). – R. MAGGIO, F. AIELLO, and A. MONROY, *Nature* 188, 1195 (1960).

² V. R. POTTER, *J. biol. Chem.* 169, 17 (1947). – A. L. LEHNINGER, *J. biol. Chem.* 178, 625 (1949). – E. C. SLATER and K. W. CLELAND, *Biochem. J.* 55, 566 (1953).



% inhibition of oxidative phosphorylation (●—●) and of cytochrome oxidase activity (▲—▲) of rat liver mitochondria caused by varying amounts of sea water. Technical details as described in the text.

Vergleichende Elektronenmikroskopie reiner DNS und der DNS des Bakteriennukleoids

Die Diskussion um die Existenz eines Bakterienkernes und dessen Struktur war schon lange vor den ersten erfolgreichen Ultradünnschnitten¹ äusserst rege²⁻⁴. Die Frage nach der makromolekularen Ultrastruktur erfuhr durch die recht verschiedenartigen Ergebnisse und Schlussfolgerungen der einzelnen elektronenoptisch arbeitenden Laboratorien eine neue Belebung^{5,6}.

Fasst man die stark divergierenden Hypothesen und Deutungen der Befunde in zwei Gruppen zusammen, so stehen Ansichten, in Anlehnung an die Chromosomenstrukturen höherer Organismen, über ein oder mehrere kompakte Bakterienchromosomen, die in einem elektronenoptisch nicht darstellbaren «Kernsaft» schwimmen sollen⁷⁻⁹ und zum Teil als recht komplexe «Großschrauben» (GIESBRECHT^{7,8}) gedeutet werden, diametral den Befunden und Hypothesen der zweiten Gruppe gegenüber¹⁰. KELLENBERGER et al. zeigen, dass unter bestimmten definierten Fixierungsbedingungen eine von keiner Membran begrenzte, mit homogenem, feinem DNS-Plasma ausgefüllte Kernvakuole beobachtet werden kann. Abweichungen von diesen Bedingungen führen zu elektronenoptisch dichten Körpern, die von den Autoren als Koagulate betrachtet werden. Argumente für diese Folgerungen waren jedoch indirekter Natur¹⁰.

kynase or of glucose-6-phosphate (G-6-P)-dehydrogenase used in the determination of the esterified P, was ruled out.

When working with homogenates of sea urchin eggs, therefore, it appears important to be able to check the amount of contaminating sea water. We have found that the most convenient way to do this is the estimation of Na^+ . It is known in fact that the Na^+ content of sea urchin³ and starfish egg⁴ is very small, the prevailing ion being K^+ . Therefore, from the estimate of Na^+ in any preparation, the amount of sea water present can be calculated quite accurately. In our practice Na^+ has been determined by flame-photometry.

Our test system consisted of a preparation of rat liver mitochondria; cytochrome oxidase has been estimated manometrically as described by MAGGIO and MONROY¹ and esterified P was determined enzymatically as G-6-P by the use of the dehydrogenase⁵.

Riassunto. Piccole quantità di acqua di mare causano una forte inibizione della fosforilazione ossidativa, mentre l'effetto sulla attività citocromossidasi è piuttosto modesto. L'ione responsabile di questo effetto è il Calcio.

FRANCA AIELLO and RACHELE MAGGIO

Laboratory of Comparative Anatomy, The University of Palermo (Italy), April 28, 1961.

³ Lord ROTHSCHILD and H. BARNES, J. exp. Biol. 30, 534 (1953).

⁴ A. TYLER, A. MONROY, C. Y. KAO, and H. GRUNDFEST, Biol. Bull. 111, 153 (1956).

⁵ This work has been supported in part by a grant of the National Institute of Health, U.S. Public Health Service (RG-6211) to the Laboratory of Comparative Anatomy.

Das Problem kann jedoch unmittelbar angegriffen werden, wenn gezeigt werden kann, dass reine DNS sich gegenüber den Fixierungsbedingungen gleich oder ähnlich verhält wie das DNS-haltige Plasma des Bakterienkernes. Soll reine DNS oder die Bakterien-DNS im Nukleoid im Dünnschnitt dargestellt werden, so ist zu bedenken, dass erst nach einer Fixierung und Dehydratisierung in üblichen Einbettungsmitteln eingebettet werden kann. Was ist bei diesen Vorgängen zu erwarten? Die DNS ist physi-

¹ G. B. CHAPMAN and J. HILLIER, J. Bact. 66, 362 (1953).

² B. DELAPORTE, Rev. gén. bot. 51, 615, 689, 748 (1939).

³ G. PIEKARSKI, Erg. Hyg. Bakteriell. Immunitätsf. exp. Therap. 26, 333 (1949).

⁴ C. F. ROBINOW, in addendum zu «The Bacterial Cell», von R. J. Dubos (Harvard University Press, Cambridge 1945), p. 355.

⁵ A. RYTER und E. KELLENBERGER, Z. Naturforschung 13b, 597 (1958), hier ausführliches Schrifttum.

⁶ R. G. E. MURRAY, Ausführlicher Beitrag in: The Bacteria, vol. I, The Internal Structure of Cell (Academic Press Inc., London 1960), p. 35.

⁷ P. GIESBRECHT, Naturwiss. 45, 473 (1958).

⁸ P. GIESBRECHT und G. PIEKARSKI, Archiv Mikrobiol. 31, 68 (1958).

⁹ E. D. DELAMATER, Ann. Rev. Microbiol. 8, 23 (1954).

¹⁰ E. KELLENBERGER, in Microbial Genetics, 10th Symposia of the Society for General Microbiology (University Press, Cambridge 1960).

kalisch-chemisch ein Kolloid mit den Eigenschaften hochpolymerer mehrbasischer Säuren^{11,12}. Wird nun ein lyophiles Kolloid durch Mischen mit Alkohol oder Aceton entwässert, so werden mit abnehmendem Wassergehalt mehr und mehr die das Kolloid stabil erhaltenden Ladungen entfernt. Schliesslich bricht das Kolloid zusammen, es fällt aus. Wir geben im Folgenden einige Resultate wieder, die im Rahmen von Untersuchungen über den Mechanismus der Fixierung erhalten wurden.

Material und Methode. *Escherichia coli* B, gezüchtet in aminosäurefreiem synthetischem Milieu M 9⁵, Kulturen belüftet. – Fixierungslösungen: 1prozentige OsO₄-Lösung in Veronalazetat-Michaelis-Puffer pH 6,2 oder pH 7,2, jeweils mit oder ohne Ca⁺⁺ in Form von CaCl₂. – Versen-Lösung (Dinatrium-ethylen-diamintetrazetat), 0,25 molar in Veronalazetat-Michaelis-Puffer ohne Ca⁺⁺. – DNS-Präparate: (1) Präparat aus Forellensperma, Dr. ZAHN, Institut für vegetative Physiologie der Johann-Wolfgang-Goethe-Universität zu Frankfurt¹³. (2) DNA highly polymerized der Nutritional Biochemicals Corporation Cleveland, Ontario, USA. (3) DNS aus Kalbsthymus nach der Guanidinmethode, Institut für Virusforschung, Heidelberg. – Dialysiermembran: einfaches Haushaltszellophan «Zellopak». – Veronalagar als Umhüllungsmittel: 3prozentiger Difco-Pulver-Agar in Michaelis-Veronalazetat-Puffer pH 6,2 oder pH 7,2. Nach Erhitzen auf 100° abgekühlt auf 45°, behält er eine gewisse Zeit seine flüssige Phase bei. Bakterien und DNS werden bei 45° eingemischt. Nach Erstarrung der Mischung können die Blöcke der üblichen Form (1/2 mm Kantenlänge) geschnitten werden. – Kunststoffeinbettung in Vestopal W⁵. – Mikrotom: Ultratome der LKB-Producter Stockholm, mit Glasmessern ausgerüstet. – Mikroskop: Siemens Elmiskop I, 60 kV, Kondensor I + II, Kondensorblende 200 μ , Strahlenverkleinerung auf 5 μ , Objektivaperturblende 30 μ , Aufnahmen auf Typonfilm FCK.

Resultate. Umhüllt man reine DNS als hochvisköses farbloses Kolloid, gelöst in Veronalazetat-Michaelis-Puffer, in Mengen von 0,05–0,01 ml mit einer Dialysiermembran, so bildet man damit, bildlich gesprochen, einen «künstlichen Kern». Diesen künstlichen Kern unterwarfen wir den gleichen Fixations- und Dehydratisierungsbedingungen, die für die Bakterien gelten, und erhielten für die Fixationsbedingung pH 6,2, Anwesenheit von Ca⁺⁺, mit oder

ohne nachfolgende 2stündige «Waschung» mit Uranylazetat⁵ im Dünnschnitt ein feinfädiges Bild einer nur schwach koagulierten DNS mit Fäden von 25–60 AE im Durchmesser (Figur 1). Fixierten wir bei einem pH von 7,2 und entfernten wir restliche Ca⁺⁺ durch eine Versen-Waschung, so zeigten die Dünnschnitte grob koagulierte recht kompakte Strukturen der DNS, wie in Figur 2 erkennbar. Wir gingen nun einen Schritt weiter und fixierten reine visköse DNS zusammen mit Bakterien. Um das Kolloid der reinen DNS zusammen mit den mechanisch stabilen Bakterienzellen fixieren zu können, mussten aus der logarithmischen Wachstumsphase abzentrifugierte Bakterien als Sediment zusammen mit viskös-dickflüssigen reiner DNS mit dem elektronenoptisch kontrastlosen Umhüllungskolloid Agar-Agar vermischt werden. Die Agarblöcke wurden in üblicher Weise über Nacht in der Fixationslösung belassen, danach entweder sofort in der Acetonreihe dehydratisiert oder zuvor noch mit Uranylazetat oder Versenlösung 2 h gewaschen. Danach Entwässerung und Vestopaleinbettung wie üblich. Die Dünnschnitte zeigen hier entsprechend dem pH-Wert der Fixationslösung und entsprechend der Nachbehandlung in der Kernvakuole der Bakterien wie ausserhalb der Bakterienzellen die simultan entwässerte DNS in äusserst ähnlicher Struktur, sowohl im Hinblick auf den Fadendurchmesser wie auch auf die Anordnung der Fadengruppen. Figur 3 zeigt den Aspekt einer feinfädigen DNS, intrazellulär wie auch extrazellulär, mit Fadendurchmesser von 25–60 AE, Figur 4 einen Ausschnitt von Figur 3, höher vergrössert. Dieses Ergebnis konnten wir bei jeder Fixationsreihe erhalten, sofern wir bei einem pH von 6,2 mit Ca⁺⁺ in der Fixierlösung arbeiteten oder anschliessend mit Uranylazetat das DNS-Kolloid stabilisierten. In diesem Fall ist die Anwesenheit von Ca⁺⁺ nicht erforderlich. Fixierten wir bei pH 6,2 oder 7,2, entfernten wir alle Ca-Ionen durch Versen und verzichteten auf eine nachfolgende Uranylazetatbehandlung, so ergaben sich grobkoagulierte DNS

¹¹ K. H. MEYER, *Natural and Synthetic High Polymers* (Interscience Publishers Ltd., London 1950), p. 255.

¹² E. CHARGAFF und I. N. DAVIDSON, *The Nucleic Acids* (Academic Press Inc., London 1960), vol. III, p. 15.

¹³ Für Überlassung eines reinen DNS-Präparates aus Forellensperma sind wir Herrn Dr. ZAHN sehr zu Dank verpflichtet.

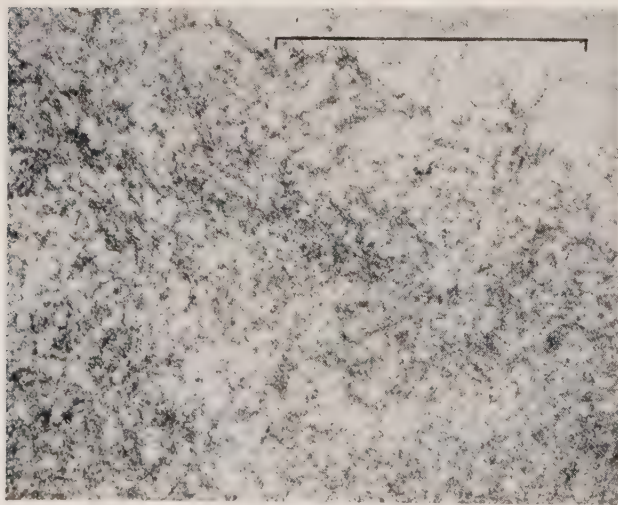


Fig. 1. DNS, von Dialysiermembran umhüllt, OsO₄-Fixierung bei Anwesenheit von Ca⁺⁺ im Fixans, pH 6,2. Feinfädige Strukturen, 25–60 AE messend. Vergr. 11000:1.



Fig. 2. DNS, von Dialysiermembran umhüllt, OsO₄-Fixierung, keine Ca⁺⁺ im Fixans, anschliessend 2stündige Versen-Behandlung. Grob koagulierte Strukturen. Vergr. 42000:1.

Strukturen in vielgestaltiger, mehr oder weniger kompakter Form, wobei wiederum die Bakterien-DNS in der Kernvakuole wie auch die reine DNS ausserhalb der Bakterienzellen sehr ähnliche Konfigurationen zeigten (Figuren 5 und 6).

Zusammenfassend können wir sagen, dass sich bei gleichen Präparationsbedingungen reine DNS-Kolloide verschiedenster Herkunft (Forellensperma, Kalbsthymus) völlig gleich verhalten in ihrer Antwort auf die präparativen Eingriffe wie die DNS in der Kernvakuole der Bakterien. Das feinfädige Bild der DNS, wie es Figur 1, 3 und 4 zeigen, entspricht einigermaßen dem, was man sich unter einer kolloidalen Lösung vorstellt. Dagegen wird doch sicherlich der Aspekt in Figur 2, aber auch in Figur 5 und Figur 6 wohl von jedermann als eine Koagulation angesprochen.

Es muss sich die Bakterien-DNS des Nukleoids in einem physikalisch-chemischen Zustand befinden, der nicht weit entfernt sein kann von demjenigen reiner DNS. Ob und in

welcher Form an die DNS gebundene Histone und Protamine das Resultat beeinflussen, wird gegenwärtig untersucht. Auf den Organisationszustand der Bakterien-DNS kann hier nicht im einzelnen eingegangen werden. In der Mehrzahl der Fälle erscheint das feinfädige DNS-Plasma bündelförmig angeordnet^{8,14,15}. Auf Grund dieser und zahlreicher weiterer Beobachtungen wurde ein Modell des Bakterienchromosoms entwickelt¹⁰, das sowohl den genetischen wie auch den physikalischen und morphologischen Befunden gerecht wird. Es ist daraus ersichtlich, dass es nicht notwendig ist, die komplexe Organisation höherer Chromosomen auf die Bakterien zu übertragen. Dennoch ist das Modell nicht als endgültig zu betrachten, da zum Beispiel zum gegenwärtigen Zeitpunkt die Frage nach der

¹⁴ W. VAN ITERSSEN und C. F. ROBINOW, J. biophys. biochem. Cytol. 9, 171 (1961).

¹⁵ A. M. GLAUERT, E. M. BRIEGER und J. M. ALLEN, Exp. Cell Res. 22, 73 (1961).

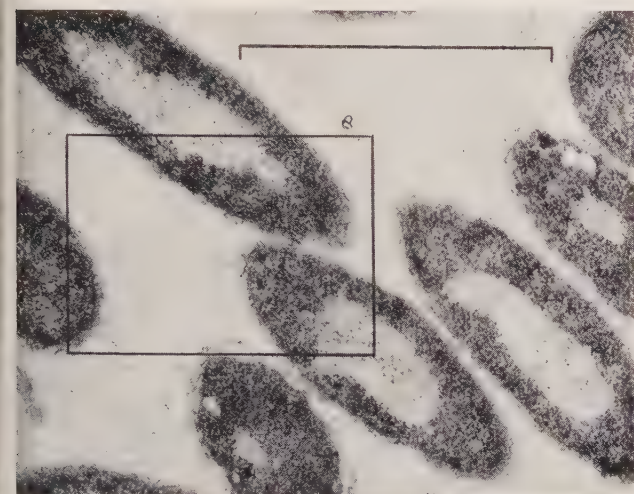


Fig. 3. *E. coli* B, zusammen mit reiner DNS von Agar-Agar umhüllt. OsO₄-Fixierung bei Anwesenheit von Ca⁺⁺ im Fixans. Aspekt einer feinfädigen (nur schwach koagulierten) intra- und extrazellulären DNS, Fadendurchmesser 25–60 AE. Vergr. 41000:1.

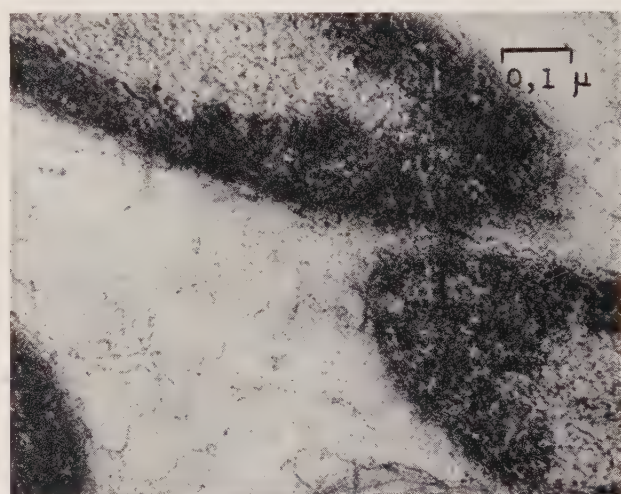


Fig. 4. Ausschnittvergrößerung von Figur 3. Vergr. 95000:1.

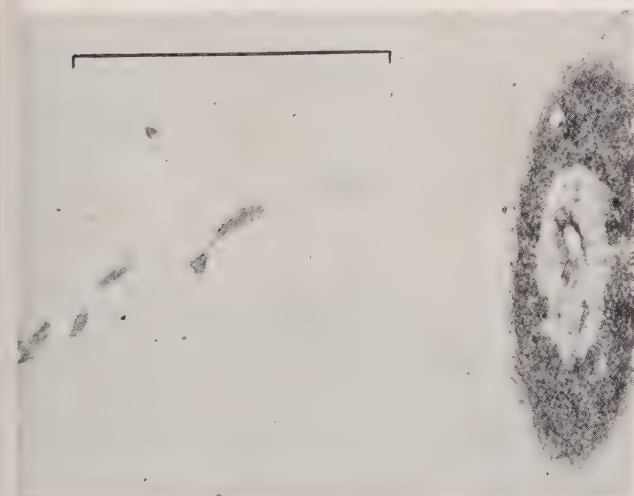


Fig. 5. *E. coli* B, zusammen mit reiner DNS von Agar-Agar umhüllt. OsO₄-Fixierung ohne Ca⁺⁺, Versen-Behandlung. Grobkoagulierte Strukturen, äusserst ähnliche Konfigurationen. Vergr. 42000:1.

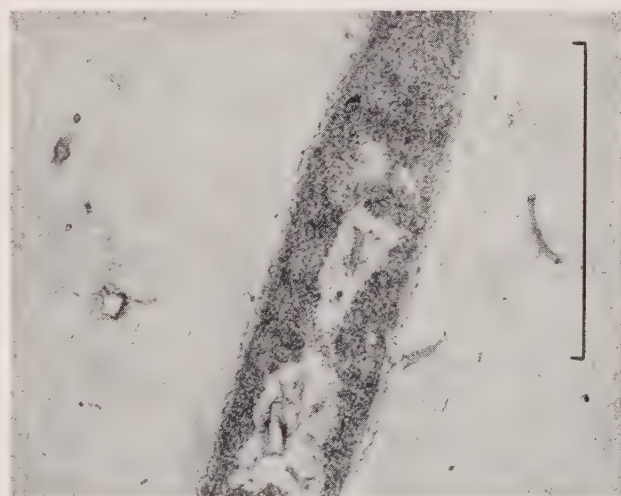


Fig. 6. *E. coli* B, gleiche Fixationsverhältnisse wie Figur 5. Man beachte die typischen Koagulationsstrukturen mit einer optisch fast leeren Innenzone. Extrazellulär wie intrazellulär gleiches Verhalten der DNS. Vergr. 42000:1.

Länge des DNS-Moleküls noch nicht beantwortet werden kann. Aus diesen Beobachtungen, zusammen mit früheren und anderen noch nicht mitgeteilten, ergibt sich, dass die Osmiumfixierung noch nicht das DNS-Kolloid oder das DNS-Plasma «fixiert» im Sinne einer Verfestigung, sondern gewissermaßen *die Weiche stellt* für die Vorgänge, die später bei der Entwässerung stattfinden. Es wurde von KELLENBERGER¹⁶ die Forderung aufgestellt, dass das DNS-Kolloid so vorzubehandeln sei, dass bei der Dehydratisierung Vernetzungen entstehen. Dadurch geliert das Kolloid zuerst, was zur Folge hat, dass die nachfolgende Koagulation, beziehungsweise das Aneinanderlagern der Fadenmoleküle auf ein Minimum beschränkt werden kann.

Zusätzliche Einzelheiten dieser Arbeiten und nachfolgende Diskussionen der Resultate werden an anderer Stelle mitgeteilt werden¹⁷.

Summary. The behaviour of pure isolated DNA towards the conditions of fixation and dehydration—prior to ultrathin sectioning—has been studied and compared with

the behaviour of the DNA-plasm of the bacterial nucleus. It is found that those conditions which produce coarse coagulation of the free DNA also produce electron opaque bodies of variable aspect inside the bacterial nucleus. The coagulation figures obtained in both situations are very similar. This is a further direct evidence in favour of the view that the bacterial nucleus is an assembly of fine DNA-containing fibrilla in a highly hydrated plasma.

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Laboratoire de Biophysique, Université de Genève (Suisse), 8. Juni 1961.

¹⁶ E. KELLENBERGER, in *Advances in Virus Research* (Academic Press Inc., London 1961), im Druck.

¹⁷ Vorliegende Arbeit wurde mit der Unterstützung des Schweizerischen Nationalfonds durchgeführt. HANNA RUHE und SOPHIA ANZEN sei für wertvolle Mitarbeit gedankt. E. KELLENBERGER danke ich für Einführung in Arbeitsgebiet und Problemstellung sowie wertvolle Diskussionen.

'Pitfalls' in Immune Electron Microscopy

Immune electron microscopy¹ is an extension of immunofluorescence² at the subcellular level. Not only have many of the problems of immunofluorescence been inherited, but it has become apparent that other problems exist. Some of the problems encountered are presented. It is hoped that other problems can be predicted early in the development of the technique, to provide a more orderly and realistic approach than was encountered in immunofluorescence.

Methods and Results. Fixation. Unfixed and 10% formalin-fixed chorioallantoic membrane and normal mouse liver, kidney, heart, and spleen were treated with an immune *Staphylococcus aureus* ferritin conjugate¹ for 1 h; the treated material was then thoroughly washed and the unfixed material fixed in 10% formalin. The specimens were embedded in paraffin and 4 μ -sections stained with Gomori's iron stain³. As demonstrated by the iron stain, the tissues fixed prior to treatment with the immune conjugate exhibited a marked diminution of ferritin as compared with the tissues fixed subsequent to treatment with the conjugate.

Treatment on Copper Grids. Bacterial, viral, and mycotic agents were fixed in 10% formalin and drops placed on formvar and carbon covered grids⁴. The grids were air-dried, specific immune conjugate applied, then washed several times in deionized water. Electron microscopy revealed that even the most thorough washing failed to remove all of the ferritin conjugate not bound by the immune reaction.

Non-specific Reactions. Horse spleen ferritin⁵ was diluted with phosphate buffered saline, pH 7.2, to contain 10 mg/ml of protein and conjugated with fluorescein isothiocyanate⁶. 0.5 ml of the ferritin fluorescein conjugate was injected intraperitoneally into mature mice. Sections of liver, embedded in paraffin, revealed that the ferritin-fluorescein conjugate was phagocytized by the Kupffer cells non-specifically (i.e. in regard to immune reactions). Observations were made using an ultraviolet light source and filters previously outlined⁶.

Discussion. The proper use of fixatives is of great importance in the detection of antigens by immune electron microscopy. Penetration of antibody into cellular material is dependent upon adequate fixation. Correlation can be

made with immunofluorescence; specific fluorescence is generally observed in the periphery of unfixed cells, while in fixed preparations the immune reaction can be observed in the periphery of the nuclear membrane. These observations are consistent with those of HIRAMOTO et al.⁷.

Fixation prior to treatment with the ferritin conjugate prevents pinocytosis, resulting in a marked diminution of the unreacted conjugate as compared with material fixed after treatment with the conjugate. Formalin generally fulfills the criteria as an excellent fixative for this technique. It does not tend to alter the antigenic components is germicidal for most pathogens, and preserves the cellular architecture. Fixatives generally used in electron microscopy are potent oxidants, and thorough screening should be made to determine their effect on the antigenic system to be used. This does not preclude the use of these oxidants to enhance the architecture if applied subsequent to the immune reaction.

In the detection of sites of toxin activity and *in vivo* experiments, the use of adequate controls cannot be too greatly stressed. Ferritin can be observed under the electron microscope in normal cellular material (HAMPTON⁸, KUFF and DALTON⁹, and BESSIS and BRETON-GORIUS¹⁰) as well as ferritin conjugate which has been non-specifically phagocytized by the reticulo-endothelial system. Therefore, in correlation with nonimmune conjugate controls, histochemical methods, fluorescein-labeled ferritin and electron microscopy should be utilized. These will not only provide a control of the immune

¹ C. W. SMITH, J. F. METZGER, S. I. ZACKS, and A. KASE, *Proc. Soc. exp. Biol. Med.* **104**, 336 (1960).

² A. H. COONS and M. H. KAPLAN, *J. exp. Med.* **91**, 1 (1950).

³ F. B. MALLORY, *Pathological Technique*, (W. B. Saunders Co., Phila. 1942), p. 137.

⁴ E. RIBI, W. BROWN, and G. GOODE, *J. Bact.* **79**, 142 (1960).

⁵ S. GRANICK and L. MICHAELIS, *J. biol. Chem.* **147**, 91 (1943).

⁶ J. D. MARSHALL, W. C. EVELAND, and C. W. SMITH, *Proc. Soc. exp. Biol. Med.* **98**, 898 (1958).

⁷ R. HIRAMOTO, M. N. GOLDSTEIN, and D. PRESSMAN, *J. Nat. Cancer Inst.* **24**, 255 (1960).

⁸ J. C. HAMPTON, *Blood*, **15**, 480 (1960).

⁹ E. L. KUFF and A. J. DALTON, *J. Ultrastructure Res.* **1**, 62 (1957).

¹⁰ M. C. BESSIS and J. BRETON-GORIUS, *J. biophys. biochem. Cytol.* **3**, 503 (1957).

reaction, but as well detect any ferritin that is present due to pinocytosis or non-specific phagocytosis.

The electrostatic action of copper grids generally tends to attract ferritin molecules non-specifically; therefore it is not desirable to use the direct application of the immune ferritin conjugate on the grid as unreacted ferritin is present in the background. Although some degree of specificity can be determined, in critical evaluations the unreacted ferritin can give rise to erroneous interpretations of antigen-antibody reactions at the subcellular level. Fragments of antigens separated from encapsulated organisms can also present these results. Therefore direct application of the conjugate on copper grids should generally be used for screening procedures, while for critical evaluations thorough washing and embedding should be used.

Immune electron microscopy presents a method of detecting antigens at the submicroscopic level as well as providing a method for the study of pathogenesis at the subcellular level. When used with caution, adequate controls, and a realization of the problems of immunology and technique involved, it will no doubt provide information which has been heretofore only conjecture. As new and more sensitive immunologic techniques are evolved, advancements must be made to balance sensi-

tivity against specificity. An equal effort should be made to provide immune sera with higher degrees of specificity, taking into consideration the immune response desired and the sensitivity of the immune technique involved. The 'pitfalls' presented should not discourage the use of ferritin conjugates in the detection of immune reactions, but only point out some problems involved. As advancements are made in electron microscopy and immunology, there is no doubt that a more effective method of conjugation will become available which will provide a more critical evaluation of the detection of antigen-antibody reactions at the subcellular level.

Zusammenfassung. Es werden die Fehlerquellen diskutiert, die bei der Untersuchung von Antigenen auftreten, wenn zu deren elektronenmikroskopischer Darstellung Antikörper, markiert mit Ferritin, verwendet werden. Durch geeignete Wahl der Fixierung können einige dieser Schwierigkeiten beseitigt werden.

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Bacteriology, Immunology and Infectious Disease Branch, Armed Forces Institute of Pathology, Washington (D.C.), March 21, 1961.

Cytodifferentiation Induced by Ribonucleoproteins

In previous research¹, we were able to demonstrate that ribonucleoproteins (RNP), extracted from different chicken organs following Niu's² method, induce changes in the development of the chick embryo rudiments or of the tissue cultures. Recently, we succeeded in improving these results. Our new findings will be presented in this paper³.

RNP were extracted from heart (RNP_H) and liver (RNP_L) of adult chicken (often white Leghorn cock). The solution in Gey liquid of these RNP was prepared at a concentration corresponding to an absorption of 5.0 at 260 mμ in the Beckman spectrophotometer (1 cm cell) (i.e. diluting 1:10, the absorption was 0.5). This was the concentration used. Instead, only Gey fluid (Holtfreter

fluid when the RNP was from frog, see below) was used in the controls. Shaken egg albumen was coagulated in hot water, and a piece 4 × 2 × 1 mm in size was then dried on filter paper under sterile conditions, imbibed with the Gey fluid or with the RNP solution. The imbibed pieces of coagulated albumen were grafted into the chorioallantois of a chick embryo of 8 days old. The shell was closed again with a coverslip and was opened after 5 days. The chorioallantois with the grafted albumen was then fixed in Bouin fluid, imbedded into paraffin and finally sectioned. The experimental procedure is summarized in Figure 1.

¹ S. RANZI, G. GAVAROSI, and P. CITTERIO, Istituto Lombardo (Rend. Sci.) B 94, 254 (1960).

² M. C. NIU, Proc. Nat. Acad. Sci. Washington 44, 1264 (1958).

³ This investigation was supported by a grant from the Consiglio Nazionale delle Ricerche.

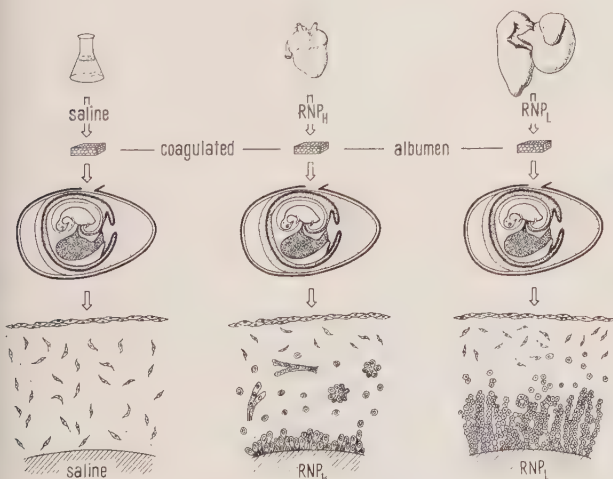


Fig. 1. Experimental procedure.

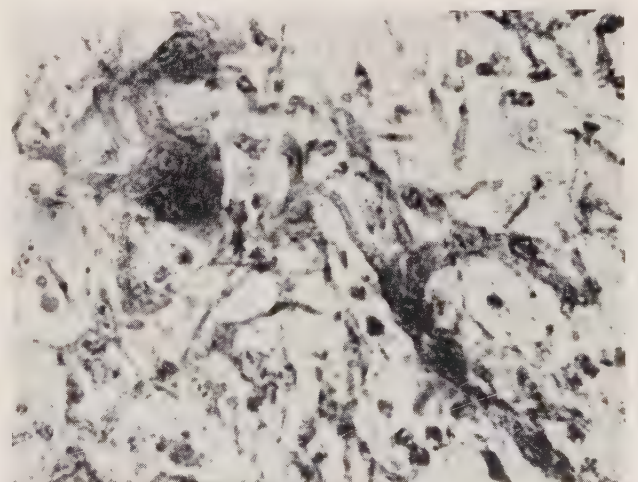


Fig. 2. Muscular elements induced by heart ribonucleoprotein (× 790).

The graft of egg albumen imbibed with saline (Gey solution or Holtfreter solution) does not induce any important change in chorioallantoic membrane, while the graft of egg albumen containing RNP_H induces the appearance of many flask-shaped or elliptical cells with intensely stainable cytoplasm and some multinucleate muscular fibers with fibrillar cytoplasm (Figure 2). We may therefore conclude that RNP extracted from heart can induce the formation of muscular fibers. The graft of egg albumen with RNP_L can induce the transformation of the epithelial cells adherent to the grafted albumen into characteristic tissue made up by polygonal cells piled up into parallel cords; some of these cells may be free in the mesenchymatic tissue (Figure 3). Consequently we may conclude that RNP extracted from liver induces the formation of liver cells.

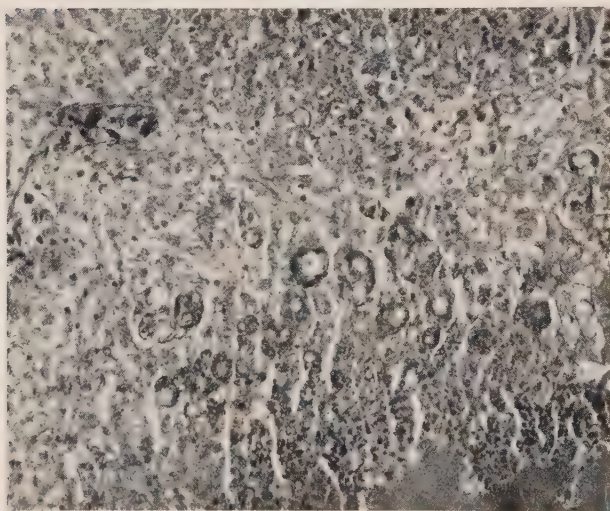


Fig. 3. Glandular structures induced by liver ribonucleoprotein. The graft is at the bottom of the picture ($\times 274$).

The Heterogeneity of Serum Glutamic-oxalacetic Transaminase of Certain Mammalian Species

Several enzymes have proved to be heterogeneous when analysed by electrophoresis or chromatography¹. These molecular forms (isozymes) of an enzyme, being similar in enzymic activity, exhibit characteristic patterns of distribution in each organ of an organism. During a study on esterases and transaminases in blood plasma (serum) of pigs with hepatic lesions², the electrophoretic pattern of certain sera from pigs with high glutamic-oxalacetic transaminase (GOT) activity was characterized by two fractions with GOT activity. Meanwhile, FLEISHER et al.³ reported two fractions with GOT activity in crude extracts of heart and liver (man, dog, pig); after completion of the present work, the same authors⁴ announced their observation of two GOT-active fractions in serum of dogs following acute injury of the liver. In addition, JUNGNER⁵ reported briefly that, depending on the pH used on electrophoresis, GOT activity is detected in two or three fractions of human serum. The heterogeneity of serum GOT of certain other mammalian species will be briefly reported.

We have studied also the action of RNP extracted from frog (*Rana esculenta*) liver and we have been able to show that this RNP is able to induce, in the chorioallantois of chick embryo, the same kind of tissue induced by the RNP extracted from the chicken liver.

After having imbibed a piece of albumen with chicken RNP, we heated it in order to denaturate the RNP, putting the whole thing in a test tube in boiling water for 10 min before implanting it in the chorioallantois. The amount of induced tissues was enormously reduced and only few or no cells were transformed.

We may therefore conclude: some thermolabile substances which are able to induce organospecific cell differentiation, are present in our RNP preparations. Our RNP preparations are therefore organospecific: i.e. the heart RNP induces muscular structures; the liver RNP induces glandular tissue. The organospecificity is more effective than the speciespecificity: i.e. frog liver RNP can induce in the chick embryo the same tissue induced by the chicken RNP_L⁴.

Riassunto. Preparati di RNP di cuore o di fegato di pollo impiantati (in albume coagulato) su membrana corioallantoidea (Figura 1) inducono, in questa, formazione rispettivamente di fibre muscolari (Figura 2) e di tessuto ghiandolare (Figura 3). Il principio attivo viene distrutto con cottura a bagno maria. RNP di fegato di rana è in grado di indurre nella membrana corioallantoidea di pollo tessuto ghiandolare identico a quello indotto da RNP estratto dal fegato di pollo.

S. RANZI, G. GAVAROSI, and P. CITTERIO

Istituto di Zoologia, Università statale, Milano (Italy), May 12, 1961.

⁴ After we sent this paper to the Editor, we found by the same method that: RNP extracted from frog heart induces the same structures induced by RNP from chick heart; RNP extracted from chick skeletal muscles induces the formation of a tissue different from that induced by RNP from heart, because the nuclei are peripheral in the so-formed structures; RNP extracted from chick kidney induces the formation of tubular structures and of some other structures tentatively interpreted as glomeruli.

Blood samples were taken from individuals with known high transaminase activity. GOT and GPT (glutamic-pyruvic transaminase) were determined according to a method described by KARMEN et al.⁶ as modified by ORDELL⁷. Transaminase activity was assayed at 340 mμ with a Beckman B spectrophotometer and expressed as $\Delta A \times 10^3/\text{min/ml}$ serum. The activity of fractionated serum after electrophoresis was similarly expressed per ml fraction.

¹ Conference on multiple molecular forms of enzymes. Ann. N.Y. Acad. Sci., in press.

² K.-B. AUGUSTINSSON, C. A. GRANT, B. OLSSON, and B. THAFVELIN, Zentr. Vet.-Med. 7, 729 (1960).

³ G. A. FLEISHER, C. S. POTTER, and K. G. WAKIM, Proc. Soc. exp. Biol. Med. 103, 229 (1960).

⁴ G. A. FLEISHER and K. G. WAKIM, Proc. Soc. exp. Biol. Med. 106, 283 (1961).

⁵ G. JUNGNER, Scand. J. clin. Lab. Invest. 10, Suppl. 31, 280 (1957).

⁶ A. KARMEN, F. WROBLEWSKI, and J. S. LADUE, J. clin. Invest. 34, 126 (1955).

⁷ R. ORDELL, Opuscula (Stockholm) 1, 14 (1956).

Electrophoretic separation of protein components of blood serum was performed in cellulose columns (1.5 cm $\times$ 50 cm, veronal buffer, pH 8.4, $I = 0.1$) at 5–11°C⁸. For each run, 1.5 ml of serum was used, the current was 30 mA provided by an applied voltage of 340 V, and the duration of runs 16 h. After the completion of electrophoresis, the liquid in the column was displaced at a rate of 10 ml/h in 1.5 ml fractions. The protein concentration of each fraction was estimated by a modified Folin procedure⁸.

The results obtained are illustrated (Figure) with serum samples from man (hepatitis), cow (puerperal paresis), horse (paralytic myoglobinaemia) and pig (hepatosis diabetica). For all four species, both GOT and GPT activities were found in the α - and β -globulin fractions. Under the experimental conditions used, GOT migrated electrophoretically at a higher rate (man, pig) in comparison with GPT, at about the same rate (cow), or at a slightly lower rate (horse). The human serum analysed contained each of the two transaminases in single fractions, GOT

between the α_2 - and β -globulin fractions and GPT in the β -globulin fraction. A similar result was obtained when electrophoresis was carried out in a phosphate buffer (pH 7.6, 5 mM). The electrophoretic pattern of cow serum showed two peaks of GOT activity, one in the β -globulin region and the other with lower electrophoretic mobility. The main GOT activity of horse serum was found in the β -globulin fraction. In addition, a second peak of low activity was observed in the α_1 -globulin region. GPT activity was found in the α_2 -globulin fraction. Analyses of a series of sera from pigs with hepatosis diabetica revealed that GOT appeared either in one peak in the β -globulin region or in two peaks, one (in the β -globulin region) being common to all samples studied and the other with lower electrophoretic mobility. In addition, certain pig sera gave a peak of low GOT activity in the albumin region. GPT was always found in a single peak migrating close to but slower than GOT.

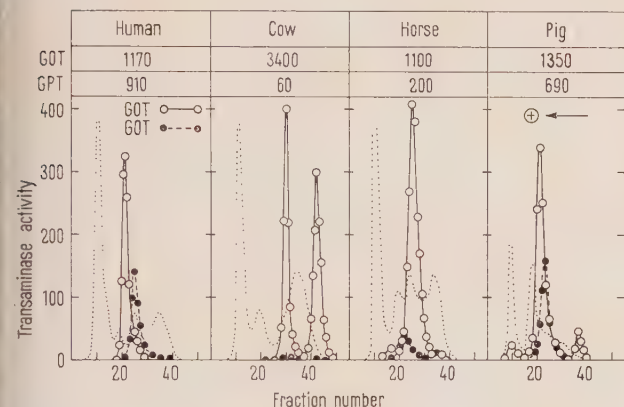
The existence of two fractions with GOT activity in certain mammalian sera may be of importance in clinical studies. If the heterogeneity of this enzyme is actually due to two different molecular forms, these may differ in relative concentration from one disease to another, e.g., in myocardial diseases and hepatic disorders. In addition, it may well be that the two forms have different cellular origin. Our experience with a series of pig sera suggests that differences in clinical course and autopsy findings are reflected in electrophoretic patterns as far as the GOT activity is concerned.

Zusammenfassung. Es wird mit Hilfe der Elektrophorese nachgewiesen, dass Glutaminsäure-Oxalessigsäure-Transaminase im Gegensatz zu Glutaminsäure-Brenztraubensäure-Transaminase in zwei verschiedenen Fraktionen des Blutserums von Kühen und Schweinen vorhanden ist.

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⁸ K.-B. AUGUSTINSSON, Acta chem. scand. 13, 571 (1959).



Electrophoretic patterns of sera from man, cow, horse, and pig. Transaminase activity expressed as $\Delta A_{340} \times 10^3/\text{min/ml}$ fraction. Relative protein contents (...) measured by the Folin colour. Figures on top of each electropherogram refer to the transaminase activity of the original sample (dialysed against the buffer used in electrophoresis).

Erhöhung der Körpertemperatur von *Periplaneta americana* L. im Verlauf zweier Bakteriosen

Die Angehörigen der Unterklasse Insecta gelten als poikilotherm¹. Für *Apis mellifica* konnte Esch² neuerdings experimentell exakt nachweisen, dass ihr Wärmehaushalt als heterotherm aufzufassen ist. Uns interessierte die grundsätzliche Frage, ob Insekten im Verlauf von Infektionskrankheiten Veränderungen der Körpertemperatur zeigen.

Als Versuchstier wählten wir *Periplaneta americana*, da hier von KÖHLER³ bereits einschlägige Beobachtungen am gesunden Tier vorliegen. GÖSSWALD⁴ berichtete unter anderem über Steigerungen der Körperinnentemperatur desselben Versuchstieres nach Insektizid-Intoxikationen, wobei beachtet werden muss, dass ein Teil des Vergiftungsablaufes von einer Exzitation des begifteten Tieres begleitet ist. Die von uns verwendeten Erreger *Serratia marcescens* Bizio und *Pseudomonas aeruginosa* Migula (Stamm CCEB 481) werden von KRIEG⁵ als fakultative Insektenpathogene kategorisiert. *Serratia marcescens* wurde bereits aus *Periplaneta americana* isoliert⁶, ausser-

dem liegen Infektionsversuche an einer nahen Verwandten, *Blatella germanica* L.⁷, vor. *Pseudomonas aeruginosa* erwies sich für andere Orthopteren bei intracoelomarer Injektion als hochgradig pathogen⁸. In Vorversuchen mit dem letztgenannten Erreger erzielten wir entsprechende Ergebnisse bei *Periplaneta americana*. Nach unseren Beobachtungen kommt es bei keiner der beiden Bakteriosen zu einer Exzitation der Versuchstiere. Vielmehr sind die Krankheitsverläufe unter anderem durch Erschlaffung der Antennen und zunehmende Akinese der Tiere gekennzeichnet. In Spätstadien sahen wir bei der durch *S. mar-*

¹ H. WEBER, *Grundriss der Insektenkunde*, 3. Auflage (1954).

² H. ESCH, Z. vgl. Physiol. 43, 305 (1960).

³ F. KÖHLER, in Vorbereitung.

⁴ K. GÖSSWALD, XI. Intern. Entomologenkongress Wien (17. bis 25. 8. 1960). "

⁵ A. KRIEG, *Grundlagen der Insektenpathologie* (1961).

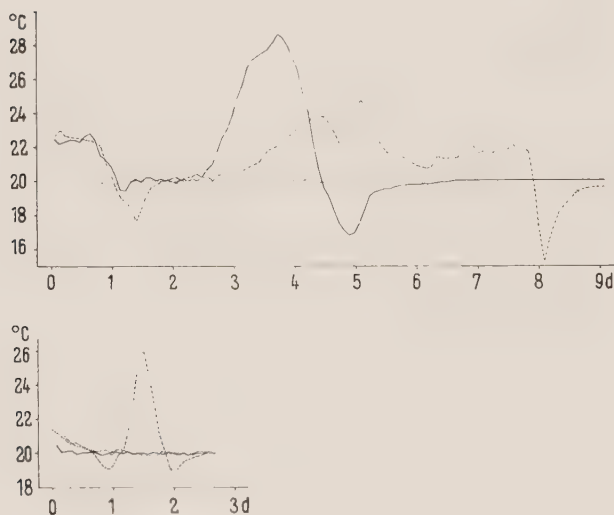
⁶ E. A. STEINHAUS, Hilgardia 28, 351 (1959).

⁷ A. M. HEIMPEL und A. S. WEST, Canad. J. Zool. 37, 169 (1959).

⁸ G. E. BUCHER und J. M. STEPHENS, Canad. J. Microbiol. 3, 611 (1957).

crescens verursachten Erkrankung gelegentlich einen gelegentlichen Extremitätentremor.

Für unsere Versuche verwendeten wir ausschliesslich männliche Imagines. Die Tiere wurden vor Versuchsbeginn bei Zimmertemperatur gehalten. Die Applikation der Erreger (kultiviert auf Merck-Standard-I-Nähragar) erfolgte unter CO_2 -Narkose intrapericardial (im Abdomen), um eine rasche Verbreitung der Bakterien im Wirt zu erreichen. Es wurde jeweils mit 1 bzw. 2 00-Insektennadeln Kultur infiziert. Auf eine genaue Dosierung verzichteten wir, da beim Punktieren des Rückengefässes häufig Hämolymphe austritt, die unkontrollierbare Erregermengen wieder ausschwemmt. Als Kontrollen dienten unbehandelte Tiere und «leer» punktierte. Während des Versuches befanden sich die Tiere in einem Thermostaten bei 20°C und 50% relativer Luftfeuchtigkeit. Sie waren einzeln an Nadeln mit Kolophoniumwachs thorakal fixiert. Spangengloben boten Laufmöglichkeit. Die Temperaturmessung erfolgte mit Hilfe von punktgeschweissten Thermoelementen (Kupfer/Konstantan). Der Durchmesser der Drähte betrug je $0,02\text{ mm}$, wobei der Messspitzendurchmesser durch Säureeinwirkung auf $50\text{ }\mu$ reduziert war. Die Meßspitze wurde in das 2. Thorakalstigma eingeführt, das Gegenthermoelement konstant bei 0°C gehalten. Ein Mehrfarben-Punkteschreiber registrierte



Verlauf der Temperatur von gesunden und infektionskranken *Periplaneta americana* (jeweils Einzeltiere). Oben: unbehandelte Kontrolle, ----- 1 Nadel *Serratia marcescens*, — 2 Nadeln *Serratia marcescens*. Unten: — unbehandelte Kontrolle, «leer» punktierte Kontrolle, ----- 2 Nadeln *Pseudomonas aeruginosa*.

Phosphodiesterase from *Thiobacillus thioparus*

During studies of the nucleolytic enzymes in extract of the autotrophic bacteria *Th. thioparus*, on the basis of identification of the products of hydrolysis of yeast ribonucleic acid (RNA) and other observations, the presence of at least three different enzymes was established, viz. 5'-nucleotidase, phosphodiesterase and ribonuclease. Ribonuclease was isolated and its properties partly investigated¹. In the present paper some properties of phosphodiesterase, which was separated from ribonuclease and partly purified, are reported.

Phosphodiesterase activity was determined, using $\text{Cabis}(p\text{-nitrophenyl})\text{-phosphate}]_2$ as substrate². The reaction

zweistündlich die Thermospannung. Vor dem Versucheichten wir die Spannungen der einzelnen Temperaturstufen ein, nach dem Versuch überprüften wir sie. Die Empfindlichkeit des Schreibers wurde so einreguliert, dass 1°C 12 mm entsprach. Die Temperaturregistrierung begann etwa 1 h *post infectionem* und erstreckte sich über maximal 10 Tage. Bei einem Teil der Versuche liessen wir zur Kontrolle die Thermostattemperatur mitschreiben. Die Versuche liefen meist an vier Tieren gleichzeitig (1 unbehandelte Kontrolle, 1 «leer» punktierte Kontrolle, 2 Versuchstiere).

Bei sämtlichen infektionskranken Versuchstieren kam es nach einer Inkubationszeit von 1–2,5 Tagen zu einer signifikanten Erhöhung der Körpertemperatur, die bis zu einer maximalen Differenz von $12,6^\circ\text{C}$ zur Umgebungstemperatur fortschreiten konnte. Wie aus der Figur (oben) zu ersehen ist, kann eine Erhöhung der Temperatur, wenn auch unter Schwankungen, bis zu einem Zeitraum von 5 Tagen beibehalten werden. Während der Inkubationsphase auftretende Temperaturunterschiede zur Umgebung vermögen wir noch nicht zu typisieren, sie sind teilweise auch bei Kontrolltieren zu beobachten. Da es durch die Narkose und sonstigen Manipulationen zu einer vorübergehenden Erregung der Tiere kommt, mögen sie zum Teil dadurch bedingt sein, zum Teil könnte es sich dabei auch um Verletzungsfolgen handeln. Die Akinese der Versuchstiere kann mit dem Höhepunkt der Temperaturkurve zusammenfallen, sie kann auch zu einem späteren Zeitpunkt erfolgen. Ein Tier, das eine Infektion mit *S. marcescens* überlebte, zeigte ebenfalls eine typische Temperaturerhöhung, die jedoch maximal lediglich $2,1^\circ\text{C}$ betrug. Später kam es bei dem Tier wieder zu einer Normalisierung der Temperatur. Die in der Figur wiedergegebenen Temperaturkurven scheinen den von uns bereits in unseren Vorversuchen beobachteten akutereren Verlauf der durch *P. aeruginosa* verursachten Bakteriose im Vergleich zu den von *S. marcescens* hervorgerufenen zu bestätigen⁹.

Summary. It is shown that two bacterial diseases of *Periplaneta americana* are accompanied by a significant increase of body temperature of the insect.

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⁹ Die verwendeten Bakterienstämme verdanken wir Herrn Prof. Dr. DIMMLING vom Institut für Hygiene und Mikrobiologie der hiesigen Universität und Herrn Dr. O. LYSSENKO vom Laboratory of Insect Pathology, Prag.

mixture contained $100\text{ }\mu\text{l}$ 0.01 M substrate in 0.1 M Tris-HCl buffer of pH 8.5, $100\text{ }\mu\text{l}$ $2 \times 10^{-3}\text{ M}$ solution of MnSO_4 and $10\text{ }\mu\text{l}$ of enzyme solution. After incubation at 37° for 60 min, the reaction was stopped by adding 2.8 ml of 0.1 N NaOH. The precipitate, if any, was centrifuged, and extinction of $p\text{-nitrophenol}$ split off was determined at $400\text{ m}\mu$ with the Uvispec spectrophotometer (Hilger Co., London). Enzyme activity was expressed in μM of $p\text{-nitrophenol}$ split off by 1 ml of enzyme solution at 37° during 60 min.

¹ W. OSTROWSKI and Z. WALCZAK, Acta biochim. Polon., in press.

² R. L. SINSHEIMER and J. F. KOERNER, J. biol. Chem. 198, 293 (1952).

The moist mass of bacteria, growing on a standard medium previously described³, was ground up with 5 vol 0.1 M citrate-phosphate buffer of pH 7.0 in a mortar with carborundum. After centrifuging, the supernatant was dialyzed overnight against distilled water and any precipitate formed was discarded. Protamine sulphate in aqueous solution was next added in amounts of 75 mg protamine/100 mg nitrogen in the extract. After centrifuging, the supernatant was then fractionated with ammonium sulphate, fraction precipitated between 0.25 to 0.55 saturation being retained. The ammonium sulphate fraction was first dialyzed against distilled water and then against 0.01 M Na-phosphate buffer of pH 7.6 and submitted to DEAE-cellulose column chromatography⁴. Elution was carried out with NaCl solutions of various concentrations buffered with 0.01 M phosphate buffer, pH 7.6. Phosphodiesterase activity was found to be concentrated chiefly in the fraction desorbed at 0.2 M NaCl at pH 7.6 (Figure 1). In peak 5 almost all 5'-nucleotidase activity present in ammonium sulphate fraction was concentrated. The fractions exhibiting the highest specific

activity of diesterase when pooled contained approximately 60% of the total activity adsorbed on the column and a 30-fold concentration as compared with crude extract. The preparation thus obtained is contaminated with 5'-nucleotidase causing hydrolysis of 5'-phosphonucleosides to the corresponding nucleosides. 3'-Phosphonucleosides are completely resistant to the action of this enzyme. The preparation of phosphodiesterase above described was used in further experiments.

Diesterase from the cells of *Th. thioparus*, in distinction to diesterase of snake venom^{5,6}, is strongly activated by Mn^{++} ions with the optimal concentration at $10^{-3} M$, when bis(*p*-nitrophenyl)-phosphate is used as substrate.

³ W. OSTROWSKI and A. KRAWCZYK, Acta biochim. Polon. **4**, 249 (1957).

⁴ H. A. SOBER and E. A. PETERSON, Fed. Proc. **17**, 1116 (1958).

⁵ G. SCHMIDT in *The Nucleic Acids* (Acad. Press Inc., Publishers, New York 1955), vol. I, p. 585.

⁶ W. E. RAZZELL and H. G. KHORANA, J. biol. Chem. **234**, 2105 (1959).

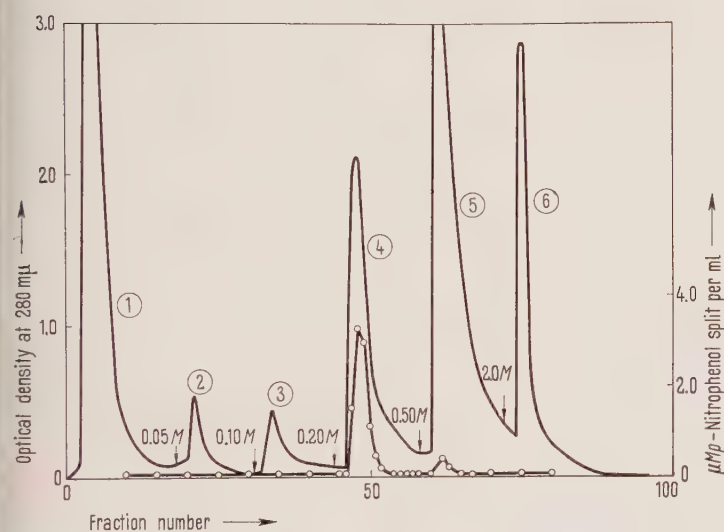


Fig. 1. Stepwise separation of ammonium sulphate fraction on DEAE-cellulose column. The sample (600 E_{280} units) and column (1.4×18 cm) were separately equilibrated with 0.01 M Na-phosphate buffer, pH 7.6. The elution was performed by passing NaCl solutions of various concentrations buffered with 0.01 M phosphate buffer, pH 7.6. Fractions (9 ml) collected at 120 ml/h, temperature 5° — absorbancy at 280 mμ, o—o phosphodiesterase activity.

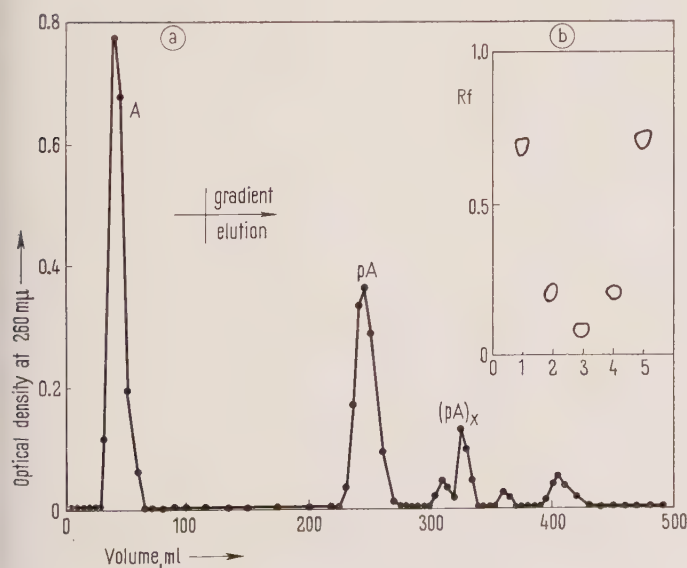


Fig. 2. (a) DEAE-cellulose chromatography of the products of hydrolysis of poly A. Poly A, 12 mg, was incubated in 0.05 M Tris-HCl buffer, pH 8.5 with 100 μ l phosphodiesterase solution of an activity of 30 μ M *p*-nitrophenol/ml in a total volume 1.0 ml for 8 h at 37° . The reaction mixture was deproteinized (isoamyl alcohol-chloroform) and chromatographed on a DEAE-cellulose column (1×18 cm). After washing the column with about 100 ml of 0.01 M NH_4HCO_3 , pH 8.6, the gradient elution was started. The gradient system consisted of a mixing chamber containing 250 ml of 0.01 M NH_4HCO_3 , pH 8.6, and from the reservoir first 0.05 M ammonium bicarbonate, then 0.5 M, pH 8.6, was slowly dropped. A flow rate of 1 ml/min was maintained and 5 ml fractions were collected. —●—● absorbancy at 260 mμ. A = adenosine, pA = adenosine-5'-phosphate, (pA)x = oligonucleotide, non-identified. — (b) Reproduction of chromatogram illustrating the R_f values of the hydrolysis products of poly A, recovered from the DEAE-cellulose column. The pooled fractions from the column were dried in vacuum, dissolved in water and chromatographed in solvent system: ethanol-1 M ammonium acetate, 75:30 v/v, pH 7.5, overnight. The spots on the filter paper were photographed in UV light. — (1) A; (2) pA; (3) (pA)x; (4) (pA)x after hydrolysis with snake venom diesterase for 1 h at pH 8.6; (5) pA after hydrolysis with 5'-nucleotidase for 1 h at pH 8.5.

In contrast to this, Zn^{++} ions at the same concentration inhibit its activity by approximately 80%. At about five-fold higher concentration of Mn^{++} , the enzyme inhibited by zinc ions regains its original activity, indicating competition between the two ions. Other bivalent metal ions possess non-specific action on the enzyme activity, either inhibition (Ca^{++} , Cu^{++}) or activation (Mg^{++} , Fe^{++} , Co^{++}), but only to a slight degree. Phosphate ions in a concentration of 0.1 M decrease the enzyme activity by 70%. In the presence of Versene (0.02 M), activity is lowered to 1/7 of that originally present. The optimum pH in the presence of Mn^{++} ions lies at about pH 9.0, and at pH 8.5 in the absence of the activator.

During electrophoresis on a starch block at pH 8.6 diesterase moves toward the anode, indicating the acid character of the enzyme. Most of the inactive proteins are separated from the diesterase activity.

The enzyme is relatively stable at alkaline pH. A loss of approximately 30% of its activity occurs when it is stored in 0.1 N NaOH at 0°, and 80% at 37° during 1 h. In an acid solution (pH less than 4.0) the enzyme is quickly inactivated. It is also thermolabile, losing 4/5 of its original activity when heated to 70° at pH 6.5 for 5 min. Appreciable loss of activity does not take place when the partially purified enzyme is stored in the frozen state (–12°) for several months and repeatedly thawed and frozen.

Phosphodiesterase causes hydrolysis of yeast RNA, RNA isolated from *Th. thioparus* cells (RNA-Th) by phenol extraction⁷, DNA from the thymus (commercial) and the 'core' of yeast RNA obtained by exhaustive digestion of RNA with pancreatic ribonuclease⁸. If one of the above-mentioned highly polymerized polynucleotides is used as the substrate, Mn^{++} ions do not activate the enzyme. The fastest rate of hydrolysis is observed with RNA-Th, and the slowest with the 'core'. The enzyme also digests synthetic polynucleotides⁹ such as polyadenylic acid (poly A) and other purine and pyrimidine 3', 5'-oligo-nucleotides. The hydrolysate of poly A was subjected to chromatography on DEAE-cellulose column¹⁰. The elution pattern of the hydrolysis products present is shown in Figure 2a. As the main products, adenosine (46%) and adenosine-5'-phosphate (31%) were found besides small amounts of oligonucleotides. The adenosine is the result of the presence of 5'-nucleotidase in the preparation of diesterase, as mentioned before. Adenosine and adenosine-5'-phosphate were identified by paper chromatography in solvent system: ethanol-ammonium acetate¹¹ and Rf values in Whatman 3 MM paper were 0.70 and 0.21 respectively. Adenosine-5'-phosphate, recovered from the column when incubated with 5'-nucleotidase isolated from the cells of *Th. thioparus*, was quantitatively transformed to adenosine (Figure 2b).

Investigations on Auxin-Gibberellin Interaction on the Growth Mechanism of *Helianthus annuus* Seedlings

The problem of auxin-gibberellin interaction on the mechanism of growth process could be discussed from two different points of view. The aim of the present research is to verify the validity of these two ideas and hence to explain the nature of this interaction on the growth of *Helianthus annuus* seedlings.

Method. The germinating achenes of *Helianthus* were sown in glass tubes containing a mixture of soil and washed sand; these were placed on a vertical klinostat under daylight. After a period of 6 days, the straight

Adenosine-3'-phosphate was completely resistant to this enzyme.

The fraction emerged from the column at higher concentrations of ammonium bicarbonate (labelled (pA)x in the Figure 2a) was first chromatographed on paper in the above solvent system (the Rf was determined to be 0.09), eluted from the paper, incubated with purified snake venom diesterase¹² and then rechromatographed under the same conditions. The spot of adenosine-5'-phosphate was only found (Figure 2b). Because the snake venom diesterase is almost without activity on oligonucleotides with 3'-phosphomonoester end groups¹³, it must be assumed that oligonucleotides formed by diesterase from *Th. thioparus* are terminated with 5'-phosphomonoester end groups.

From results reported above it can be concluded that the enzyme from *Th. thioparus* splits off the diester linkages of phosphoric acid in position 3' of polynucleotide chain. During the initial stage of the enzymic action in the presence of an excess of substrate, chiefly 5'-mononucleotides and small amounts of oligonucleotides were found. Hence, it must be assumed that phosphodiesterase causes gradual degradation of polynucleotide liberating 5'-phosphonucleotides¹⁴.

Investigations on further purification of the enzyme and its specificity are under way and will be published elsewhere.

Résumé. La phosphodiesterase a été isolée des cellules du *Th. thioparus* et partiellement purifiée. Cette enzyme hydrolyse les polynucléotides jusqu'à 5'-mononucleotides. Elle est spécifiquement activée par les ions du Mn^{++} et inhibée par les ions du Zn^{++} . Son pH optimum est compris entre 8,5–9,0. L'enzyme est thermolabile et relativement résistante en milieu alcalin.

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Department of Physiological Chemistry, Medical Academy, Cracow (Poland), February 1, 1961.

⁷ K. S. KIRBY, Biochem. J. 64, 405 (1956).

⁸ R. J. HILMOE, J. biol. Chem. 235, 2117 (1960).

⁹ The author is indebted to Dr. D. SHUGAR for a preparation of synthetic polynucleotides, poly A, poly G, and poly U.

¹⁰ M. STAEHELIN, E. A. PETERSON, and H. A. SOBER, Arch. Biochem. Biophys. 85, 289 (1959).

¹¹ R. BERGKVIST, Acta chem. scand. 11, 1465 (1957).

¹² F. FELIX, J. L. POTTER, and M. LASKOWSKI, J. biol. Chem. 235, 1150 (1960).

¹³ L. A. HEPPEL, P. J. ORTIZ, and S. OCHOA, J. biol. Chem. 229, 679, 695 (1957).

¹⁴ I wish to express my best thanks to Professor B. SKARŻYŃSKI for his help and encouragement.

hypocotyls, of length approximately 5 cm, were selected to be decapitated. The decapitation was done exactly from the base of the cotyledons which are known to serve as auxin sources to the developing plant. These seedlings were then left in complete darkness for a period of 10 h, to ensure the thorough removal of all endogenous auxins. After this procedure, capillary tubes of length 2.5 cm containing 10^{-5} Mol isomolecular solutions of indole acetic acid (IAA), naphthalene acetic acid (NAA) and gibberellin (GB) respectively, were placed on the apex of the hypocotyls. As control sets, hypocotyls bearing capillary tubes with distilled water were used. For the measurements of the combined action of GB with IAA and NAA, a mixture of equal volumes of isomolecular (10^{-5} Mol) solutions of

IAA-GB and NAA-GB were applied. The plants were placed close in front of a screen of Eidinger paper and were left in complete darkness at room temperature ($24 \pm 2^\circ\text{C}$) and relative humidity 78% for the whole of the experimental period. After having noted the initial height of the hypocotyls, measurements were taken under red light on the 4th, 6th, and 24th h, as indicated by the change in level of the reflection on the screen of the base of the capillary tubes. The values obtained were plotted against time as abscissa and straight growth as ordinate, the growth being measured as relative values of the control set. A measure of significance was performed for each exposure time in each series.

Results. The individual and the combined actions of GB with auxins are summarized in the Figure.

As the Figure demonstrates, the maximum growth reaction was produced by IAA which showed the steepest gradient compared with the other two growth regulators, GB produced a minimum action and NAA was in between. After having obtained these results, the auxin-gibberellin interaction on the same experimental object was analysed.

As seen from the Figure, the IAA-GB graph was more pronounced than the NAA-GB, yet the IAA-GB effect was nearly the same as that of IAA, for the values of both these curves do, in general, correspond. The statistical calculations revealed that the values of IAA-GB and IAA respectively were insignificant. The NAA-GB graph and the NAA-curve followed a similar path up to the 4th h, henceforth the NAA-GB graph diverged strongly to follow a parallel path with the NAA-curve but on a higher level, in accordance with the significance of the NAA-GB and NAA values after the 4th h.

Discussion. The effect of auxins and gibberellin on the growth process of plants has been subjected to much detailed research, and it was concluded that GB alone had

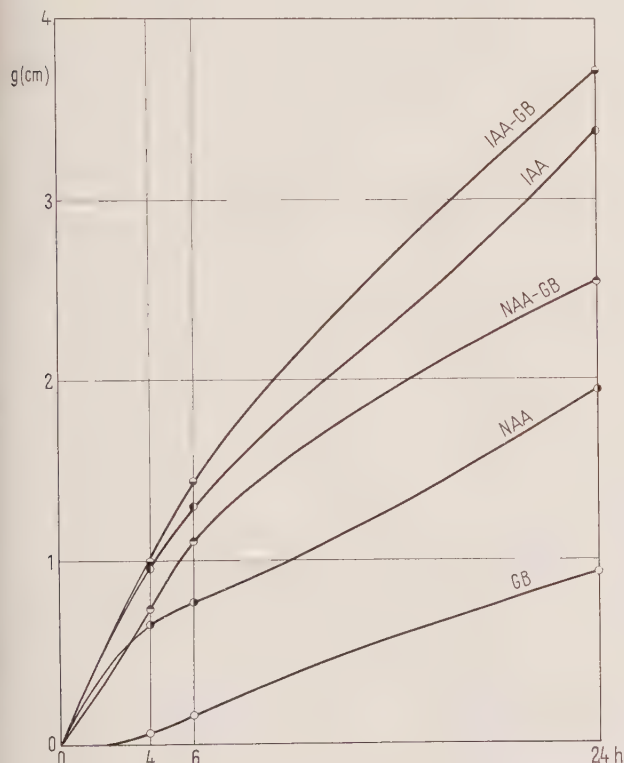
very little effect on this process. BRIAN and HEMMING¹⁻³, by applying a GB solution on the shoot of pea seedlings, found an increase in growth. KUSE⁴, in his experiments on debladed petioles of *Ipomoea Batatas*, came to the conclusion that GB applied alone produced a very slight growth, this being in good agreement with the findings of the present research. On the other hand, WAREING⁵, by applying GB on the cambial regions of woody dicotyledons, noticed very little cambial activity. This limited action of the compound on the cambial region also explains the small effect of GB on growth. As already known in the relevant literature, in the present research too, the application of NAA and especially of IAA increased the growth.

Having observed the sole effect of GB on the growth process, the auxin-gibberellin interaction attracted the attention of several research workers. Among them, HAYASHI and MURAKAMI⁶, by treating IAA-extracts of several different plants with GB, could find no increase in the conversion of tryptophan into IAA. PILET⁷ noticed the decrease in the IAA oxidase activity *in vivo*, under the effect of GB; hence he concluded that GB acts on the growth process by eliminating the IAA oxidase destruction. BRIAN and HEMMING¹⁻³ observed that, when GB is applied to pea internodes, the growth produced is the same as that of untreated plants. Hence, GB can only act in the presence of an auxin such as IAA in the ambient medium. KUSE⁴, similar to the latter investigators, noticed that GB promotes growth only in the presence of IAA. On the other hand, according to PURVES and HILLMAN⁸, the primary effects of GB and IAA are completely independent of one another, and when applied together their combined action is only partly additive. According to these different observations, the two concepts which could explain the auxin-gibberellin interaction are that: either GB acts through an auxin-mediated medium, or else the action of GB is independent of other growth regulating systems. The results of the present paper show that the auxin-gibberellin interaction cannot be interpreted fully with either of these two concepts, but that the effect of GB depends on the nature of the auxins. This is clearly shown in the results obtained, namely that whilst GB added to IAA showed practically no different effect from IAA alone, yet GB added to NAA was significantly additive compared to NAA after the 4th h⁹.

Résumé. L'auteur a étudié l'action combinée des gibberellines et des auxines chez l'hypocotyle de l'*Helianthus annuus*. L'action de IAA + GB n'a pas été différente de l'action de l'IAA seule, tandis que l'NAA + GB a eu un effet promotif que n'avait pas l'NAA seule. D'où l'on peut déduire que cette action combinée dépend de la nature de l'auxine.

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Department of General Botany, University of Istanbul (Turkey), January 9, 1961.



The effect of IAA, NAA, GB, and IAA-GB, NAA-GB on the straight growth of *Helianthus annuus* seedlings. Abscissa: time in h and growth of control set. Ordinate: straight growth in cm.

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2. P. W. BRIAN and H. G. HEMMING, *Nature* 179, 417 (1957).
3. P. W. BRIAN and H. G. HEMMING, *Ann. Bot.* 22, 1 (1958).
4. G. KUSE, *Bot. Mag.* 71, 151 (1958).
5. P. F. WAREING, *Nature* 181, 1744 (1958).
6. T. HAYASHI and Y. MURAKAMI, *Proc. 1st Japanese Gibberellin Symposium, Tokyo* (1957).
7. P. E. PILET, *C. R. Acad. Sci.* 245, 1327 (1957).
8. W. K. PURVES and W. S. HILLMAN, *Physiol. Plantarum* 12, 786 (1959).
9. In conclusion the authors wish to express their many thanks to Miss C. N. VERTER for her helpful assistance and to Professor H. TAMIYA for having kindly provided samples of gibberellin.

Formation of Histamine in Transplants from a Rat Mammary Carcinoma

A high rate of histamine formation has been recognized as part of various types of normal rapid tissue growth¹. The rat embryo, during a certain phase of growth, exhibits a high histamine forming capacity (HFC) as a result of activation of histidine decarboxylase^{2,3}. Embryonic growth can be arrested by enzyme inhibition⁴. In reparatively growing tissues of healing skin wounds, the HFC was 50–60 times the level in control skin⁵. The rate of wound healing in rats, as measured by the tensile strength of the wound, could be retarded or enhanced by measures which depressed or elevated the HFC⁵. The rate of collagen formation in healing skin wounds could be increased by elevating the HFC⁶. Extracellular histamine derived from injected 'long-acting-histamine' (histamine dipicrate) had no effect on the rate of healing or collagen formation⁶. The histamine content of these tissues with a high HFC was very low, and mast cells were largely absent. In man the histidine decarboxylase activity is higher in blood containing immature myeloid cells, capable of mitosis, than in blood where these cells are few or absent⁷.

Once the relationship between histidine decarboxylase and normal rapid tissue growth was recognized, it appeared likely that a similar association would be found also in malignant growths. Accordingly, in rats bearing a subcutaneously implanted hepatoma, a high rate of histamine formation was found which fell to normal on removal of the tumour; the histidine-decarboxylase activity was high in homogenates of hepatoma tissue and low in normal rat liver⁸.

The object of the present study was to determine the HFC of a rat cancer tissue *in vitro* by a sensitive method permitting the use of low, physiological concentrations of histidine. The tumour tissue was obtained from female albino rats bearing subcutaneous transplants of the Walker rat mammary carcinoma 256. Portions of 1 g of minced cancer tissue, after suspension in 3 ml of 0.1 M phosphate buffer of pH 7.3, were incubated with 40 µg ¹⁴C-histidine for 3 h at 37°C under nitrogen. The amount of ¹⁴C-histamine formed during the incubation was determined by isotope dilution with histamine dihydrochloride as carrier according to SCHAYER, by the standard procedures as described in detail⁹. The cancer tissue produced histamine at considerable rates. In 4 determinations on 4 separate tumours respectively 0.094, 0.096, 0.139, and 0.154 µg ¹⁴C-histamine (in terms of the

base) was formed per g tissue in 3 h. The histidine decarboxylase activity of the tumour tissue was considerably increased by the addition of pyridoxal-5-phosphate. In normal rat mammary tissue, excised from pregnant rats 20–21 days after mating, the rate of histamine formation was too low to permit exact determinations. The histamine content of the malignant and normal mammary tissue was very low, in the range of 2 µg/g (base). Mast cells were not found in the tumour tissue investigated. A similar discrepancy between high HFC and low histamine content is characteristic of embryonic and granulation tissue where the high HFC is non-mast cell in nature.

The present observations are in line with reports from this institute which suggest that rapidly formed intracellular histamine actively and locally promotes certain types of tissue growth.

Zusammenfassung. Tumorgewebe von Ratten mit subcutan transplantiertem Walker-Milchdrüsencarcinom 256 produzierte ¹⁴C-Histamin mit beträchtlicher Geschwindigkeit bei Inkubation des Krebsgewebes mit ¹⁴C-Histidin. Zusatz von Pyridoxal-5-phosphat aktivierte die Histidine-decarboxylase des Krebsgewebes. In normalem Milchdrüsengewebe lag die Histaminbildung ausserhalb des Messbereichs. Der Histamingehalt des Krebsgewebes war sehr niedrig und Mastzellen wurden keine gefunden. Der Histaminmetabolismus des Carcinoms 256 ist demjenigen im Rattenembryo und im Granulationsgewebe heilender Hautwunden ähnlich.

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¹ G. KAHLSON, *Lancet* 1960/I, 67.

² G. KAHLSON, E. ROSENGREN, and H. WESTLING, *J. Physiol.* 143, 91 (1958).

³ G. KAHLSON, E. ROSENGREN, and T. WHITE, *J. Physiol.* 151, 131 (1960).

⁴ G. KAHLSON and E. ROSENGREN, *Nature* 184, 1238 (1959).

⁵ G. KAHLSON, K. NILSSON, E. ROSENGREN, and B. ZEDERFELDT, *Lancet* 1960/II, 230.

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⁸ D. MACKAY, P. MARSHALL, and J. RILEY, *J. Physiol.* 153, 31P (1960).

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In vitro Uncoupling of Oxidative Phosphorylation in Normal Liver Mitochondria by Serum of Sarcoma-Bearing Rats

In previous reports (NANNI¹), an uncoupling of oxidative phosphorylation was observed in liver and kidney mitochondria of sarcoma-bearing rats. Treatment *in vivo* with, and addition *in vitro* of, extracts of cortical or of necrotic central parts of this tumour cause in liver mitochondria of normal animals an evident uncoupling of phosphorylations from oxidations, greater in the first case than in the second. In the case of kidney mitochondria, treatment with the two kinds of extracts does not modify oxidative phosphorylation. It would therefore seem that a substance (or several substances) forming the soluble phase of cortical zone neoplastic cells is able to act on mitochondria

containing enzymes of oxidative phosphorylation, producing an uncoupling of phosphorylation from oxidation. Work by many authors has demonstrated that neoplastic tissues give out substances which pass into the circulation (SHERMAN et al.²); FUKUOKA and NAKAHARA³ have shown a thermostable protein in the serum of sarcoma-bearing animals. Other authors demonstrated inhibitive actions on many enzymatic activities by tumour-elaborated substances (ELLIOT⁴, DIANZANI⁵, EMMELOT and

¹ G. NANNI, *Lo Sperimentale*, in press.

² C. D. SHERMAN, J. J. MORTON, and G. B. MIDER, *Canc. Res.* 10, 371 (1950).

³ F. FUKUOKA and W. NAKAHARA, *Gann* 44, 1 (1953).

⁴ K. A. C. ELLIOT, *Biochem. J.* 34, 1134 (1940).

⁵ M. U. DIANZANI, *Tumori* 36, 304 (1950).

Bos⁶); ROSE⁷ observed that the serum of sarcoma-bearing animals has a picnotic activity on HeLa cells. In the serum a hypoalbuminaemia was found in tumour-bearing animals (MIDER et al.⁸), with an increase of α_2 - and β -globulins (CLAUSEN et al.⁹). Many investigators observed an increase in plasma mucoproteins belonging to the α_2 -globulins (SEIBERT et al.¹⁰). MILLER and BERNFELD¹¹ demonstrated an anomalous protein in the plasma of C₃H mice with spontaneous sarcoma. The present investigation was undertaken to ascertain whether the substance (or substances) responsible for the uncoupling of oxidative phosphorylation in Galliera-sarcoma-bearing rats was present also in their serum.

This investigation was made on Galliera sarcoma-bearing albino rats. This is a spontaneous tumour of rats, isolated in 1920 and preserved by regular transplantation. The sarcoma appears to consist of a growing cortical zone and of a central zone, in which necrosis phenomena are predominant. Microscopically, it appears to be a polymorpha cell sarcoma (NOVELLI¹²). For the preparation of serum we used normal rats and tumour-bearing rats, 25 days after grafting. The animals were bled to death and the blood was collected in sterile test tubes, the serum was used within 24 h. Liver mitochondria were isolated, as previously described (NANNI¹), from normal albino rats, weighing 150–170 g, fed on a standard diet. Oxidative phosphorylation was determined using 0.01 M K-glutamate as substrate. O₂ uptake was measured manometrically, using Warburg flasks equipped with one side-arm, with air as gas phase, at 25°C. Inorganic orthophosphate phosphorus was determined by the method of FISKE and SUBBAROW¹³. An amount of liver mitochondria corresponding to 200 mg of fresh tissue (about 1.5 mg N) per Warburg flask, was used. When serum activity was tested, 0.2 ml normal serum, as control, or 0.2 ml serum from sarcoma-bearing rats, were added to each flask. ATPase activity was determined in normal serum and also in serum of tumour-bearing animals, according to the method of DUBOIS and POTTER¹⁴. Nitrogen estimations were made by the usual microKjeldhal technique. The standard deviation and Student's 't' test being calculated for each average.

Table I shows the data concerning the behaviour of oxidative phosphorylation in three different series of experiments: in normal liver mitochondria, the effect of the addition *in vitro* of normal rat serum or of serum from sarcoma-bearing rats to normal liver mitochondria. In the first two series of experiments, made as controls, oxidative phosphorylation was found to be normal. In investigations made with addition of sarcoma-bearing rat serum, there is an evident uncoupling of phosphorylations from oxidations in normal liver mitochondria. Also in this case, as in previous investigations (NANNI¹), the decrease in phosphorylations is accompanied by a decrease in oxidations; this decrease does not run parallel to that of phosphorylations, therefore the P:O ratios are impaired. Among the possible reasons for this behaviour, the presence in the serum of tumour-bearing rats of a type of ATPase activity, which would destroy ATP as soon as it is formed, has been considered. A decrease of ATPase has been reported from many authors (ALLARD et al.¹⁵) in hepatomas; according to others, on the contrary, it is normal (POTTER and LIEBL¹⁶). Serum from sarcoma-bearing rats was therefore examined: ATPase activity was found to be very slight, lower even than the minimum activity which is found in normal serum (Table II). It is therefore possible that serum from sarcoma-bearing rats contains a substance or several substances which are able to produce an uncoupling of oxidative phosphorylation in animal

Tab. I. Influence of addition *in vitro* of normal rat serum or of serum from sarcoma-bearing rats on oxidative phosphorylation of normal liver mitochondria

Addition	Number of experiments	μ atoms Pi	μ atoms O ₂	P:O
None	7	11.84 ± 1.17	4.32 ± 0.56	2.74 ± 0.17
Normal rat serum	7	9.53 ± 1.27	3.50 ± 0.39	2.73 ± 0.31
Sarcoma bearing rat serum	7	5.30 ± 1.35	3.26 ± 0.43	1.63 ± 0.43 ^a

(± standard deviation; the values whose difference from the normal is statistically significant ($P < 0.01$) are indicated with^a)

Tab. II. ATPase activity in normal rat serum and in sarcoma-bearing rat serum. (The values are given as μ g of P liberated in 15 min/mg of N, at 38°C, pH 7.4.)

	Number of experiments	Activity	mg N/ml
Normal rat serum	3	1.801	12.280
Sarcoma bearing rat serum	3	1.027	10.094

organs. This uncoupling agent, according to previous reports, might derive from neoplastic tissue metabolism and would have the same effect *in vivo* and *in vitro*. The observed uncoupling seems to be due to a greater decrease of phosphorylations than of oxidations, with a consequent alteration of P:O ratios. It may be thought that neoplastic cell components, through the blood circulation, act on mitochondrial membrane permeability, thereby damaging the normal action of mitochondrial enzymes in the various organs of tumour-bearing animals. We cannot at present specify the mode of action of neoplastic tissue on oxidative phosphorylation; it is possible that it acts either by releasing mitochrome from mitochondria (PULLMAN et al.¹⁷, POLIS et al.¹⁸, SACKTOR et al.¹⁹, LEHNINGER et al.²⁰, LAUDAHN²¹), or in some other way. Further work on these problems is in progress.

⁶ P. EMMELOT and C. J. BOS, *Biochem. biophys. Acta* 19, 565 (1956).

⁷ G. G. ROSE, *Canc. Res.* 18, 411 (1958).

⁸ G. B. MIDER, E. L. ALLING, and J. J. MORTON, *Cancer* 3, 56 (1950).

⁹ J. CLAUSEN, R. RASK-NIELSEN, H. E. CHRISTENSEN, and T. MUNKNER, *Canc. Res.* 20, 178 (1960).

¹⁰ F. B. SEIBERT, M. L. PFAFF, and M. V. SEIBERT, *Arch. Biochem.* 18, 279 (1948).

¹¹ E. E. MILLER and P. BERNFELD, *Canc. Res.* 20, 1149 (1960).

¹² A. NOVELLI, *Neoplasie* 8, 56 (1954).

¹³ C. H. FISKE and Y. SUBBAROW, *J. biol. Chem.* 66, 375 (1925).

¹⁴ K. P. DUBOIS and V. R. POTTER, *J. biol. Chem.* 150, 185 (1943).

¹⁵ C. ALLARD, G. DE LAMIRANDE, and A. CANTERO, *Canc. Res.* 17, 862 (1957).

¹⁶ V. R. POTTER and G. J. LIEBL, *Canc. Res.* 5, 18 (1945).

¹⁷ M. E. PULLMAN and E. RACKER, *Science* 123, 1105 (1956).

¹⁸ B. D. POLIS and H. W. SHMUKLER, *J. biol. Chem.* 227, 419 (1957).

¹⁹ B. SACKTOR, J. J. O'NEIL, and D. C. COCHRAN, *J. biol. Chem.* 233, 1233 (1958).

²⁰ A. L. LEHNINGER and L. F. REMMERT, *J. biol. Chem.* 234, 2459 (1959).

²¹ G. LAUDAHN, *Exper.* 16, 444 (1960).

Riassunto. Gli autori hanno esaminato l'influenza esercitata *in vitro* da parte del siero di ratti portatori di sarcoma Galliera sulla fosforilazione ossidativa in mitocondri di fegato normale. Dagli esperimenti effettuati risulta che il siero di portatore di tumore determina disaccoppiamento delle fosforilazioni dalle ossidazioni nei mitocondri di fegato normale. Gli autori, in riferimento a

precedenti ricerche, discutono le probabili cause di questo fenomeno dovuto ad una sostanza o sostanze liberatesi dal tessuto neoplastico.

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Some Components of Adaptive Values of Heterozygous *Drosophila willistoni* from Irradiated Natural Populations

The adaptive value (w) is the integrative result of very numerous and complex biological properties contributing toward the genotypes' relative ability to perpetuate themselves throughout the generations.

In this work we attempt to measure some of the most important components of the adaptive values of irradiated *D. willistoni*. Wild individuals have been studied from a sample taken directly from an isolated wood, Capão A, which, in the course of a year, received about 420 000 ($70\,000 \times 6F_1$) descendents of six irradiated samples (3 times 10 000 r + 3 times 5000 r) by Cobalt 60 source of γ -radiation. As non-irradiated control, we used another isolated natural population of the same region (Eldorado, Rio Grande do Sul, Brasil)^{1,2}.

We studied the percentage of hatched eggs according to daily oviposition, the *viability* as the total number of offspring from single couples in randomised culture vials³ as true reproductive potentials of their genotypes; and the *sterility* as the mean frequencies of sterile matings³. These parameters of fitness were studied within a set of specified environmental conditions, of temperature, food supply⁴ and technical details.

The most significant results can be briefly described as follows:

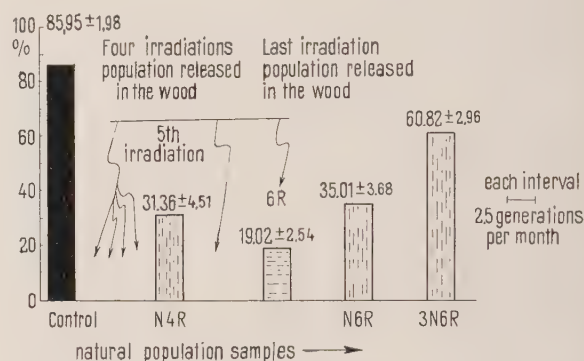
Percentage of hatched eggs was determined by counting the daily oviposition of inseminated females five days old, from the sixth to the tenth day, in all samples. The eggs were maintained at $25 \pm 1^\circ\text{C}$ at 95 to 100% of humidity. The counts of hatched (empty) eggs and the confirmation count of larvae were made 48 h after oviposition.

The first sample examined, N4R₁, was taken from Capão A one month (25 generations) after finishing a period of eight months during which four releases of 70 000 F_1 of irradiated flies (respectively $10 + 10 + 10 + 5 \text{ Kr} = 35 \text{ Kr}$) were made. In comparison with the control value (85.95 ± 1.98), the percentage of hatched eggs decreased significantly (see Figure). Two more releases of 70 000 F_1 irradiated flies ($5 + 5 \text{ Kr}$) were made during the next four months. The last irradiated population released, 6R₁, analysed also exhibited a very low percentage of hatched eggs (Figure). The increase shown after about 3 months (7.5 generations) by the N6R is most significant, however, in the 3N6R sample (3 months later), a significant three-fold increase occurred (Figure). Even this increase did not restore the level of the natural non-irradiated control population.

A total of 3280 eggs were examined in these tests.

Viability was measured by the mean number of offspring produced by fertile simple pairs of flies³. Control and irradiated natural populations, and the last laboratory irradiated sample released, were run in five simultaneous series of experiments:

(6R₁ < Control A), (N6R₁ < Control E₁), (2N6R₁ < Control E₂), (3N6R₁ = Control E₂) (6N6R₁ = Control E₄)



% of hatched eggs of *Drosophila willistoni* control and irradiated natural populations.

as can be seen in the Table. The very significant decrease of viability of the 6R₁ (–17%) was followed by the irradiated natural population samples (N6R₁ 21%; 2N6R₁ 16%) until the recuperation attained by the 3N6R₁ 6 months (15 generations) after the last release; and this was further confirmed by the 6N6R₁ 14 months (approximately 35 generations) after the 6R release.

A total of 36854 among the experimental, and 24183 individuals in the control cultures, has been counted.

The sterility defined as the number of simple pairs of flies that do not produce offspring³ gave the interesting information that in natural population they are eliminated as equivalent of dominant lethals. Only the F_1 of the 6R showed a significantly higher percentage than 37.6%. All samples from nature are around a mean value of 3.5% sterile pairs.

Some irradiated experimental population of *D. melanogaster* increased their fitness in comparison with non-irradiated³ which seems to be dependent on population size and the advantage of induced mutants in heterozygous condition. The irradiated natural populations of *D. ananassae* near the atomic bombed Marshall Islands^{5,6} showed an extreme depression in some biological properties. The flies heavily irradiated by direct and fallout radiations from atomic tests, especially in March 1954 (Bikini islands), showed a greater load of deleterious mutants and low fitness values, expressed in egg development, in comparison with much less irradiated natural

¹ H. WINGE, M. NAPP, C. M. P. MACIEL, and E. K. MARQUES, *Exper.* 17, (1961).

² A. R. CORDEIRO, *Exper.* 17, (1961).

³ B. WALLACE, *The Amer. Nat.* 872, 295 (1959).

⁴ E. K. MARQUES and C. M. P. MACIEL, *Dros. Inf. Serv.* 32, 169 (1958).

⁵ W. S. STONE and F. D. WILSON, *Univ. of Texas Publ.* 5914, 223 (1959).

⁶ T. G. GREGG, *Univ. of Texas Publ.* 5914, 207 (1959).

populations, the controls, Majuro Ponape. The return to control values occurred in 1956 and remained in 1957, 1958^{5,6}.

The experiments here reported show that the percentage of hatched eggs remains lower than the control non-irradiated populations even after the frequency of recessive lethal + semi-lethals have returned to the normal values¹.

The mean number of offspring produced by fertile single pairs, after a significant decrease recovered normal values five months after the normalization of lethal + semi-lethal frequencies¹.

The interesting results of genetical analyses, completed with allelism tests on the same populations, shows what

might be an incorporation of newly induced lethals and probably a discharge of the 'wild' lethals.

Would these newly incorporated mutants be responsible for the persistence of the physiological effects observed?⁷

Zusammenfassung. Die Analyse einiger Adaptationskomponenten (Häufigkeit des Schlüpfens aus dem Ei, Viabilität und Sterilität) bei einer isolierten natürlichen Population von *Drosophila willistoni*, zeigte nach Bestrahlung mit Co 60 verminderte Adaptationswerte, während mehrerer Generationen nach Bestrahlung, mit progressiver Angleichung an das Kontrollniveau in den folgenden Generationen. Jedoch erreichte diese Population während 15 Generationen den Grad der Häufigkeit des Schlüpfens von unbehandelten Populationen nicht.

Viability Samples	Fertile Strains	Offspring	Mean %
6 R ₁	86	2154	25.05 ± 2.13
2 EL-C-A	82	3412	41.61 ± 2.53
N 6 R ₁	197	6315	32.06 ± 1.79
El-E ₁	145	7739	53.37 ± 3.40
2 N 6 R ₁	182	3746	20.58 ± 0.77
El-E ₂	69	2542	36.84 ± 1.70
3 N 6 R ₁	207	6716	32.14 ± 1.32
6 N 6 R ₁	212	14788	69.76 ± 4.43
El-E ₄	132	10552	79.94 ± 5.19

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⁷ Work developed with grants in aid of the Conselho Nacional de Pesquisas (CNPq) (Brazilian Nat. Council of Research) Comissão Nacional de Energia Nuclear (C.N.E.N.) and the Rockefeller Foundation. – We acknowledge the constant interest and criticism of Dr. A. R. CORDEIRO in charge of the general planning of the radiation genetics project.

Chromosomal Polymorphism Decrease due to γ-Radiation on Natural Populations of *Drosophila willistoni*

Studies on genetics¹ and fitness² of isolated natural populations, irradiated for one year and compared with control populations of the same region, accompanied these cytological observations.

A short account is given of the most significant results obtained. Before irradiating the first sample from the chosen 'Capão A', the inversion frequencies of the chromosomes from the salivary glands were determined.

As mentioned in ¹, a total of about 45 kr γ-radiation was delivered in one year throughout the successive releases of F₁ flies from three samples that received 10 kr followed by three samples that received 5 kr (about 70 000 individuals in each sample). The population size determined experimentally was 3 to 10 times lower, according to the season. One month after each release the samples: N1R, N2R, N3R, N4R, N5R, and N6R were collected from Capão A simultaneously with the controls (see Tables I and II). The total cytological analysis includes about 3000 individuals. The samples N6R to 6N6R complete this analysis to 14 months after the last release: 6R₁.

For the second chromosome inversions (Table I) and the III chromosome C inversion, the pooled values from the N1R to the N5R show significantly lower frequencies in comparison with Control A, obtained from the same wood before radiation. The III C was below the Control values until the 5N6R and the IIL: D, E, and F until the 6N6R.

The mean number of inversions per individual (only females were considered) after remaining almost unchanged for the long period of seven months, from N1R to N4R, decreased significantly afterwards from the N5R to the 6N6R sample (Table II).

Another result was the detection of two new inversions restricted to the irradiated wood. One is a long sub-distal inversion in the III chromosome, the other is subproximal, short, in the XL Chromosome, called respectively R E1 and I E1. The former (III R·E1) involves the inversion of the sections 92 to the middle of 100, and the latter

Tab. I. Significant differences among various samples of *D. willistoni* natural populations, irradiated and control (X² and P)

Chromosomes and inversion	N1R–N5R	N6R	3N6R	4N6R	5N6R	6N6R
II L–D	28.10 <0.01	14.70 <0.01	15.11 <0.01	44.47 <0.01	6.25 <0.02	28.64 <0.01
II L–E	28.24 <0.01	6.91 <0.01	6.27 <0.02	37.42 <0.01	16.37 <0.01	15.72 <0.01
II L–F	30.38 <0.01	5.77 <0.02	4.63 <0.05	1.75 <0.20	6.54 <0.02	15.29 <0.01
II L–H	12.73 <0.05	7.95 <0.01	—	6.99 <0.01	—	—
III B	—	—	4.73 <0.05	—	—	—
III C	14.06 <0.05	12.43 <0.01	9.43 <0.01	5.30 <0.05	8.49 <0.01	—
III J	—	5.37 <0.02	2.71 <0.10	—	—	—
Number of studied individuals						
Irradiated	568	188	106	199	162	244
Control	133	117	117	214		240

¹ H. WINGE, M. NAPP, C. M. P. MACIEL, and E. K. MARQUES, *Exper.* 17, (1961).

² E. K. MARQUES and C. M. P. MACIEL, *Exper.* 17, (1961).

Tab. II. Mean number of inversion per individual *D. willistoni* from natural irradiated and control populations.

Irradiated natural population			Control population
N1R + N2R	3.08 ± 0.15	=	Control A
N3R	2.67 ± 0.17	<	3.59 ± 0.15
N4R	3.03 ± 0.16	=	
N5R	2.79 ± 0.09	<	
N6R	2.61 ± 0.09	<	4.14 ± 0.17
3N6R	3.35 ± 0.17	<	
4N6R	2.17 ± 0.08	<	3.26 ± 0.09
5N6R	2.49 ± 0.10	<	3.26 ± 0.10
6N6R	2.53 ± 0.11	<	

(XLI·E1) inverts the block comprising the distal half of section 4 to the end of 6 in the reference map³. No other chromosomal aberrations were observed.

In experimental *D. melanogaster* irradiated populations, PAGET⁴ observed some new inversions in high frequency. Nevertheless, SEECOF⁵ detected no increase of new rearrangements in *D. ananassae* heavily irradiated natural populations.

The adaptive advantage of heterozygous for inversions in *Drosophila*, demonstrated by the valuable work of DOBZHANSKY and many others, would, in the *willistoni* irradiated natural populations here studied, represent a protection for lethals and semi-lethals accumulated¹.

The crossing over suppression effect of inversions will favour this 'incorporation'. These trapped and protected lethals or deleterious mutants may correspond to the

interesting allelic affinities discovered in the same populations¹⁻⁶.

The decrease of inversions frequency in irradiated natural population might be due to their overload with deleterious mutants.

Zusammenfassung. In einer isolierten natürlichen Population von *Drosophila willistoni* wurden innert 7 Monaten viermal je 70 000 γ -bestrahlte Fliegen freigelassen. Anschließend wurde eine beträchtliche Abnahme der mittleren Inversionshäufigkeit pro Individuum festgestellt. Einige Inversionen im 2. Chromosom und III C, III D, III E und III F waren sogar seltener als in der nicht bestrahlten natürlichen Kontrollpopulation, selbst 14 Monate (= 35 Generationen) nach dem letzten Freilassen. Doch wurden in der bestrahlten Population auch 2 für diese Art neue Inversionen gefunden (eine im X- und eine im 3. Chromosom).

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Department of Genetics, I. C. N., Universidade do Rio Grande do Sul, Porto Alegre (Brazil), January 30, 1961.

³ TH. DOBZHANSKY, J. Heredity 41, 156 (1950).

⁴ O. E. PAGET, Amer. Nat. 88, 105 (1954).

⁵ W. S. STONE, R. WHEELER, W. P. SPENCER, F. D. WILSON, J. T. NEUENSCHWANDER, T. G. GREGG, R. L. SEECOF, and C. L. WARD, Univ. Texas Publ. 5721, 260 (1957).

⁶ Work supported in part by grants of the Rockefeller Foundation, Conselho Nacional de Pesquisas (Brazilian Research Council) and Comissão Nacional de Energia Nuclear (Braz. Atomic Energy Com.).

Genetic Effects of γ -Radiation on Natural Populations of *Drosophila willistoni*¹

As radiation levels in our planet increase, the importance and urgency of knowing its effects on natural populations, becomes more evident. Several valuable experiments have been done with *Drosophila* population cages by WALLACE^{2,3} and others, but only recently STONE et al.⁴, STONE and WILSON^{5,6}, started comparative studies on natural populations of *D. ananassae* from Bikini and other less irradiated islands. Amongst *D. willistoni*'s advantages are: the possibility of obtaining homozygous strains (II and III chrom.) by a method similar to ClB using DOBZHANSKY's marked stocks⁷, and consequently, of having exact data on the frequency and allelism of lethal, semi-lethal, sterility and visible mutants; the easy-growth in laboratory and abundance in wild environments; the low dispersion rate⁸ that limits its populations to the isolated 'capões' (island of woods in the grassland regions of Rio Grande do Sul State) etc.

A short report is here presented showing the most significant results obtained after a three-year period (1957–1960) of studies on genetic effects of γ -radiation, exhibited by an isolated natural population of *D. willistoni* inhabiting a specially chosen 'capão A' and compared with a control natural population from another 'capão' in the same region^{9,10}.

To start the experiment 1200 *willistoni* individuals were collected from the 'capão A' and bred in laboratory by daily culture bottle transfers. During one year, six doses, three of approximately 10 000 r and three of about 5000 r, were given to each alternating generation of about 15 000 individuals each time. These were bred again to be released

in the 'capão A' (about 70 000 flies) and to maintain a large laboratory stock. As IVES¹¹ demonstrated, γ -radiation produces an average increase of 2% lethal mutations per 1000 r in the dosage range of 300 r to 12 500 r. The decrease of fecundity or fertility by high doses of Cobalt 60 γ -radiation seems to be less severe than the X-rays.

Each of these releases had about 10 to 3 times the experimentally determined number of adult flies living in the 'capão'. The last laboratory irradiated 'generation': 6R₁ was sampled before the release and genetically analysed. Four samples from 'capão A' were also analysed: the N6R, 3N6R, 4N6R, and 5N6R, respectively: 2, 6, 7, and 10 months after the last release of irradiated flies (6R₁).

¹ Work developed (1957–1960) with grants in aid of the Conselho Nacional de Pesquisas (CNPq: Brazilian Nat. Council of Research) and the Rockefeller Foundation.

We acknowledge the constant interest and suggestions of Dr. A. R. CORDEIRO in charge of the general planning of the radiation genetics project.

² B. WALLACE, J. Genetics 54, 280 (1956).

³ B. WALLACE, Amer. Naturalist 93, 295 (1959).

⁴ W. S. STONE, M. R. WHEELER, W. P. SPENCER, F. D. WILSON, J. T. NEUENSCHWANDER, T. G. GREGG, and R. L. SEECOF, Univ. Texas Publ. 5721, 260 (1957).

⁵ W. S. STONE and F. D. WILSON, Proc. Nat. Acad. Sci. 44, 565 (1958).

⁶ W. S. STONE and F. D. WILSON, Univ. Texas Publ. 5914, 223 (1959).

⁷ B. SPASSKY and TH. DOBZHANSKY, Heredity 4, 201 (1950).

⁸ H. BURLA, A. B. DA CUNHA, A. G. L. CAVALCANTI, TH. DOBZHANSKY, and C. PAVAN, Ecology 31, 393 (1950).

⁹ A. R. CORDEIRO, Exper. 17, 405 (1961).

¹⁰ E. K. MARQUES and C. M. P. MACIEL, Exper. 17, 404 (1961).

¹¹ P. T. IVES, Proc. Nat. Acad. Sci. 45, 188 (1959).

Sterility tests were made separately for the homozygous males (479: II and 732: III chromosomes) and the homozygous females (489: II and 811: III chromosomes). The unexpected results of these very carefully performed tests was that no increase of such 'recessive' sterility, induced by radiation, could be detected. The 6R₁ stock had the same level of the control non-irradiated natural population (II chrom.: males $X^2 = 1.13$ $P < 0.30$; females $X^2 = 0.04$ $P < 0.90$, both: $X^2 = 0.42$ $P < 0.70$; III chrom.: males $X^2 = 2.77$ $P < 0.10$, females $X^2 = 0.34$ $P < 0.70$; both: none). This observation suggests to us that there is only a small number of *loci* that can produce recessive sterility mutants. It is also possible that these mutants are near maximal frequency in natural populations, compatible with a very efficient selection pressure over the heterozygous condition. There is, nevertheless, evidence that significant oscillations in natural populations do occur (males of 5N6R of II chrom. $X^2 = 12.80$ $P < 0.01$).

This class of mutants deserves further study.

The frequency of lethals and semi-lethals (Table I) increased significantly in the laboratory stock 6R₁ (II chrom. $X^2 = 38.17$ $P < 0.01$ and III chrom. $X^2 = 15.19$ $P < 0.01$). Nevertheless, five generations after the last release, a sample from the irradiated 'capão A' (N6R) showed only slight but no significant difference for the II chromosome, and no increase at all for the III chromosomes (Table I).

Comparable results of such elimination were observed by STONE and WILSON^{5,6} for *D. ananassae*, and for a sample of about 10 lethals of *D. willistoni* introduced in natural population by DA CUNHA et al.¹². According to CORDEIRO¹³ and CORDEIRO and DOBZHANSKY¹⁴, even the spontaneous natural population lethals of *D. willistoni* have semidominant effects, depressing, on the average, the viability of the heterozygous bearing individuals. Nevertheless, some combinations of lethal/normal chromosomes were shown to be highly viable. From this it is conceivable that all sorts of semidominant effects will be produced by newly induced 'recessive' lethals if a large sample of such mutations is studied.

However, an important discovery appeared when the allelism tests of intra and inter samples of 6R₁, N6R, 3N6R, and control were made. The high frequency of allelism intra the 6R, and the N6R samples differing significantly from controls was accompanied by a high frequency of inter 6R × N6R allelism, both for the II and the III chromosomes (Table II and III). This interesting fact shows that 5 generations after the last release, the irradiated natural population, even though having 'normal' frequency of lethals + semi-lethals, incorporated many such new radiation-induced mutants (Table II and III). Up to date, a total of 11 674 crosses have been made for the II chromosome (12 726 will be possible) and 8102 crosses for the III chrom. (10 155 possible). The sample 3N6R, taken 15 generations after the last irradiated release, is significantly different ($P < 0.01$) from 6R₁ and N6R with regard to the amount of allelism.

The inter-allelic frequency of 3N6R × control is slightly greater than the 6R₁ × control and N6R × control, but this difference is not significant considering the numbers of crosses obtained. More interesting is the fact that 3N6R has as much alleles with the 6R₁ and N6R (6R₁ × 3N6R) and (N6R × 3N6R) as with the *controls* which greatly differ from the 6R₁ and N6R. Furthermore, the allelism of (6R₁ × 3N6R) is due to different *loci* from those observed among the (control × 3N6R) crosses. These observations indicate that, 15 generations after discontinuing the invasion of the population by irradiated individuals, its effect has left detectable vestiges.

Tab. I. Frequencies, in %, of lethals + semi-lethals among II or III homozygous chromosome strains from natural population of *Drosophila willistoni* irradiated by a Co 60 source, compared with non irradiated ones.

Samples analysed	II Chromosomes		III Chromosomes	
	Number tested	Lethals + semi-lethals	Number tested	Lethals + semi-lethals
Last laboratory irradiated population (6R ₁)	119	77.31 ± 3.84	145	47.59 ± 4.15
Natural population	N6R 99	51.51 ± 5.02	162	32.10 ± 3.67
after irradiation	3N6R 147	40.81 ± 4.05	150	31.33 ± 3.79
	4N6R 101	51.48 ± 4.97	41	26.83 ± 6.92
	5N6R 107	45.79 ± 4.96	139	30.94 ± 3.92
Controls	224	42.41 ± 3.30	389	29.56 ± 2.31

Tab. II. II Chromosome of *D. willistoni*'s frequency of allelism, in %, and the number of crosses intra and inter the samples. Righthand column shows the frequencies of lethals + semi-lethals for comparison purposes.

Samples	Last irradiated 6R ₁	Natural populations		Controls	Lethals + semi-lethals
6R ₁	6.14% 1 009				77.31 ± 3.84
N6R	4.01% 1 520	3.57% 588			51.51 ± 5.02
3N6R	0.46% 1 499	0.64% 1 255	0.46% 648		40.81 ± 4.05
Controls	0.12% 1 622	0.15% 1 319	0.55% 1 456	0.79% 758	42.41 ± 3.30

Tab. III. III Chromosome *D. willistoni*'s allelism frequency, in %, and the number of crosses intra and inter the samples. Righthand column shows the frequencies of lethals and semi-lethals for comparison purposes.

Samples	Last irradiated 6R ₁	Natural populations		Controls	Lethals + semi-lethals
6R ₁	3.88% 309				47.59 ± 4.15
N6R	2.94% 781	3.07% 522			32.10 ± 3.67
3N6R	0.31% 642	0.12% 856	0.00% 373		31.33 ± 3.79
Controls	0.00% 1 034	0.07% 1 435	0.16% 1 240	0.22% 910	29.56 ± 2.31

Summing up, the return to lethal equilibrium frequency in irradiated natural populations is attained quickly, probably by selection against heterozygous bearing individuals. However there is a partial incorporation of new lethals and discharge of 'wild' ones demonstrated here,

¹² A. B. DA CUNHA, J. S. TOLEDO, C. PAVAN, H. L. DE SOUZA, and S. A. TOLEDO, Communication to the I. Simpósio Sul-Americano de Genética (São Paulo 1960).
¹³ A. R. CORDEIRO, Proc. Nat. Acad. Sci. 38, 471 (1952).
¹⁴ A. R. CORDEIRO and TH. DOBZHANSKY, Amer. Nat. 88, 75 (1954).

for the first time, throughout the allelism tests. A further step is reached when allelism frequency drops down to control values, also. Nevertheless, there are indications that a small amount of newly induced lethals remains incorporated even when all these frequencies appear to be equal to non-irradiated populations.

Zusammenfassung. Eine isolierte natürliche Population von *Drosophila willistoni* erhielt während eines Jahres eine beträchtliche Beimischung von F₁-Co 60-bestrahlten Fliegen. Die genetische Analyse ergab, dass die Häufigkeit der letalen und semiletalen Allele nach 5 Generationen rasch wieder auf das Normale sank. Die Frequenz der

Letalallele war allerdings noch hoch, gleich hoch wie in der in Massenkultur gehaltenen bestrahlten Zucht. Erst nach 15 Generationen sank sie zum Niveau der natürlichen Kontrollpopulation. Einzelne durch Bestrahlung erhaltene Letalallele blieben in der Population erhalten.

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Paradoxical Findings in Ouchterlony Tests

Ouchterlony's 'reaction of identity' in agar gel diffusion precipitation tests is sometimes misleading¹; whenever such a reaction is obtained, it should be carefully evaluated. Diffusion of precipitins present in extracts of *Ricinus communis* or *Abrus precatorius* seeds, and in horse anti-serum specific for type XIV pneumococcus, against a 1/1000 (w/v) aqueous solution of purified type XIV pneumococcus polysaccharide results² in the formation of a single continuous precipitation line (Figure 1); therefore it may be concluded that the precipitins in the horse serum and the seed extracts are identical. However, in view of the heterogenous origin of the precipitating reagents, the 'reaction of identity' should not be accepted entirely at its face value.

Extracts of *Ricinus communis* or *Abrus precatorius* seeds agglutinate the erythrocytes of many animal species and form precipitates with their sera³; it is believed that the structure which reacts with the seed principle is present both on the erythrocyte surface, and, in soluble form, in the sera of these animals; one such animal is the horse. Thus it would appear that horse anti-XIV serum contains both a precipitin identical with the seed precipitins, as well as the structure specifically precipitated by the seed reagents. This paradox requires explanation.

The 'reaction of identity' (Figure 1) is obtained by using horse anti-XIV serum in a dilution at which it does

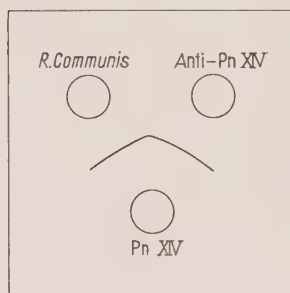


Fig. 1

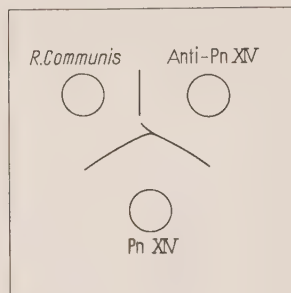


Fig. 2

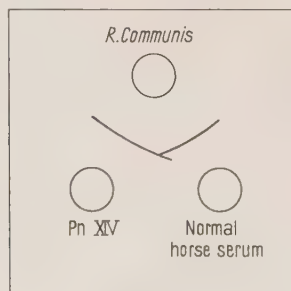


Fig. 3

not form precipitates with the seed extracts, but can still strongly precipitate the type XIV polysaccharide. When the undiluted serum is used instead, a 'reaction of partial identity' is obtained (Figure 2); this probably occurs because the precipitate formed by the pneumococcus polysaccharide and the anti-XIV serum is slightly deflected in an effort to join up with the precipitate formed by the seed extract and the antiserum. In view of the fact that the two precipitating reagents react with one another, it would be better not to attempt to interpret the precipitation pattern obtained under these circumstances (Figure 2), but to accept the 'reaction of identity' which was obtained when the two precipitating reagents did not precipitate each other (Figure 1). Moreover, it can only be claimed that the 'reaction of identity' indicates merely that the seed and serum precipitins precipitate the same molecules, and not that the precipitins are chemically identical.

The 'precipitinogen' in horse serum is in fact related to type XIV pneumococcus polysaccharide. When a sample of normal horse serum, which does not contain anti-XIV antibodies, is allowed to diffuse against the type XIV polysaccharide, and either *Ricinus communis* or *Abrus precatorius* seed extracts, a 'reaction of partial identity' is obtained (Figure 3). Nevertheless, anti-XIV antibodies appear in horse serum after immunisation with pneumococcus type XIV; they are thus highly specific and probably combine with some part of the type XIV pneumococcus polysaccharide molecule which is not common to the partially-related substance in horse serum. The seed precipitins probably combine with another part of the type XIV polysaccharide molecule. Whatever the explanation may be, the precipitins of *Ricinus communis* and *Abrus precatorius* seeds, when considered in relation to a system of pneumococcal polysaccharides, appear to be specific for type XIV pneumococcus polysaccharide². Extracts of *Ricinus communis* and *Abrus precatorius* seeds could thus be used in selected serological studies as substitutes for horse antipneumococcus XIV sera.

Zusammenfassung. Mit Präzipitationsversuchen im Agargel wird gezeigt, dass Präzipitine aus *Ricinus communis*- oder *Abrus precatorius*-Samen und die spezifischen Anti-Pn XIV-Präzipitine des Pferdeserums mit verschiedenen Seitenästen der Strukturformel von Pn XIV-Kapselpolysaccharid reagieren können.

G. W. G. BIRD

Armed Forces Medical College, Poona (India), December 21, 1960.

¹ J. G. FEINBERG, Int. Arch. Allergy, N. Y., 11, 129 (1957).

² G. W. G. BIRD, Nature 187, 415 (1960); Exper. 17, 71 (1961).

³ R. KRAUS, cited by W. W. FORD, Zbl. Bakt. I Abt. Ref. 58, 129 (1913).

The Qualitative Effect of Anesthetic Gases on Spontaneous Potentials from Explants of Brain Tissue in Culture¹

The spontaneity, duration, form, and repetitive pattern of the potentials arising spontaneously from adult and embryonic tissue explants of brain tissue in culture² are different from the multitude of previous reports of evoked and 'spontaneous' potential from cerebral tissues. These differences raise the question as to the cells of origin of the potentials arising spontaneously from explants of brain tissue in culture. By both light and electron microscopy, the explants giving rise to spontaneous potentials in culture have always contained 'normal' neuronal cells as well as the other elements usually found in nervous tissues. However the mere physical presence of neuronal cells in the explants does not necessarily mean that the potentials arose from these cells. In addition to the histological evidence, other experimental approaches have been used to try to determine the properties and hence the nature of the cells responsible for the spontaneous potentials. The study reported here concerns the general qualitative

response of the spontaneous potentials from explants of the telencephalon of 14 day chick embryos to exposure to anesthetic gases.

The explants were made in the usual way onto the upper aspect of a piece of cellulose sponge on a coverglass in such a way as to lie between the sponge and a 36 gauge platinum electrode. The reference electrode (also of 36 gauge platinum wire) was cemented onto the side of a Kahn tube into which the coverglass with the sponge, explant, and electrode were placed. A supernatant made from balanced salt solution TDL1³ and 0.25% human serum protein at 37°C was added to the Kahn tube in such a way as to come half way up the cellulose sponge and immerse the lower end of the reference electrode but not to touch the explant itself (except insofar as the supernatant permeated the cellulose sponge). The Kahn tube was sealed with a

¹ This research was supported by a grant from the Office of Naval Research, U.S. Navy Contract NONR 1598 (04).

² A. W. B. CUNNINGHAM and B. J. RYLANDER, *J. Neurophysiol.* **24**, 141 (1961).

³ A. W. B. CUNNINGHAM, M. DOUGHERTY, and B. J. RYLANDER, *Nature (Lond.)* **186**, 477 (1960).

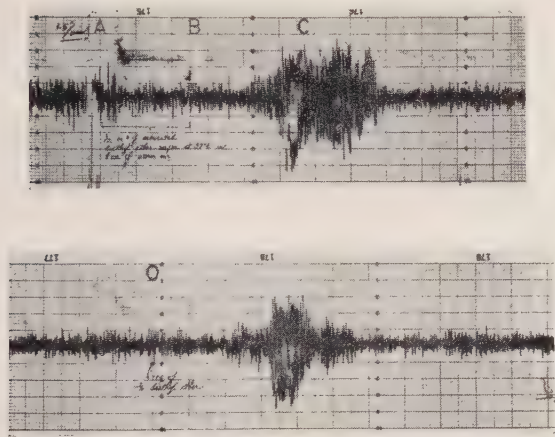


Fig. 1. The excitatory effect of small doses of anesthetic ($1/10 \text{ cm}^3$ of air saturated with di-ethyl ether vapor in 8 cm^3 warm air injected into 7 cm^3 Kahn tube) on the spontaneous potentials from explants of chick embryo telencephalon. Lower trace is a direct continuation of the upper one. From A to B—slow injection of anesthetic vapor. C start of excitatory effect. O—second injection of anesthetic vapor ($1/2 \text{ cm}^3$ of air saturated with di-ethyl ether vapor injected into 7 cm^3 Kahn tube). X axis—each division equals 4 sec. Y axis—eight large divisions equals $30 \mu\text{V}$.

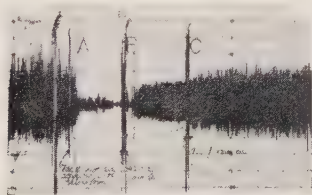


Fig. 2. The effect of chloroform vapor (1 cm^3 of air saturated with chloroform vapor at 37°C injected into 7 cm^3 Kahn tube) on spontaneous potentials from an explant of chick embryo telencephalon. A—point of injection of chloroform vapor. B and C—points of injection of warm air to 'wash out' chloroform vapor. X axis—each division equals 8 sec. Y axis—eight large divisions equals $30 \mu\text{V}$.

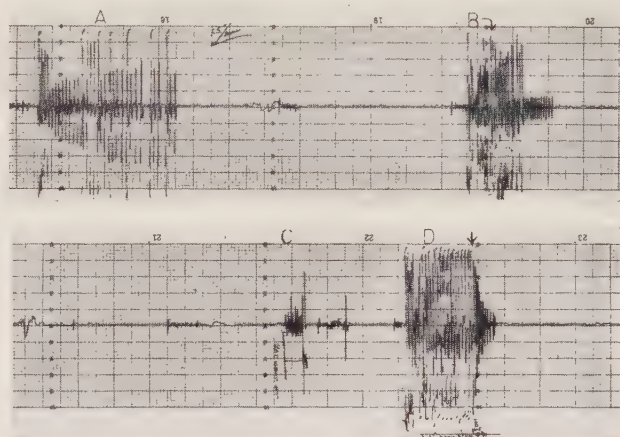


Fig. 3. The effect of di-ethyl ether vapor on the spontaneous potentials from explants of chick embryo telencephalon. Lower trace is the direct continuation of the upper one. A—normal series of potentials, B— 1 cm^3 of $1/8$ di-ethyl ether vapor (air saturated with anesthetic vapor at 37°C diluted with more air) administered at the arrow of this series, C—point at which di-ethyl ether vapor displaced by warm air, D series of potentials still showing some 'excitement'. At the end of this series (indicated by arrow) 1 cm^3 di-ethyl ether vapor was administered. X axis—each division equals 4 sec. Y axis—eight large divisions equals $30 \mu\text{V}$.

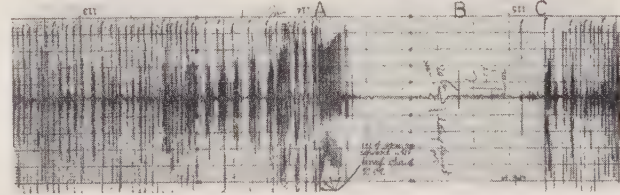


Fig. 4. The effects of di-vinyl ether vapor (1 cm^3 of air saturated with di-vinyl ether vapor at 37°C injected into 7 cm^3 Kahn tube) on spontaneous potentials from explants of chick telencephalon. A—point of administration of anesthetic vapor, B—start of injection of warm air to displace anesthetic vapor, C—recommencement of spontaneous potentials. X axis—each division equals 4 sec. Y axis—eight large divisions equals $30 \mu\text{V}$.

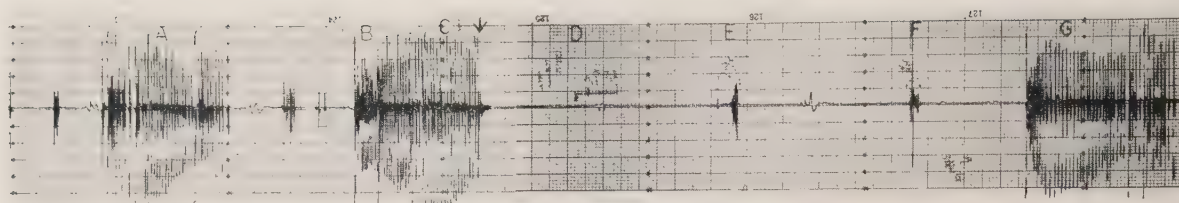


Fig. 5. The effect of halothane vapor on spontaneous potentials from an explant of chick embryo telencephalic tissue in culture. The lower trace is a direct continuation of the upper trace. A and B are normal series of potentials. At the arrow at C, a dose of halothane vapor (1 cm³ air saturated with halothane vapor at 37°C) is administered and then washed out repeatedly with warm air at D, E, and F. G is the point of return of the spontaneous potentials. X axis — each division equals 1 sec. Y axis — eight large divisions equals 30 μ V.

serum stopper containing an air filter (to prevent any change in the pressure inside the Kahn tube) and a length of Teflon tubing to allow the introduction of the anesthetic gases. The stopper was inserted so as to allow the egress of the two platinum electrodes. The Kahn tube was placed in a shielded incubator at 37°C and connected by shielded cables to amplifiers and a paper strip recorder.

An injection of a volume of warm air similar to that already in the Kahn tube caused only a transient incidental electrical disturbance as can be seen at the time of injection in any of the illustrations. The anesthetic gas to be used was firstly carefully warmed to 37°C before injection by syringe into the outer end of a piece of Teflon tubing. The anesthetics used were: Chloroform (Figure 2), di-ethyl ether (Figure 3), divinyl ether (Figure 4), and Halothane (Figure 5). Each of the anesthetics had the same general effect. They caused an initial short period of increased frequency of potential production—a stage of 'excitement' which is best seen in Figure 1 where low concentrations of anesthetics were used. It is also visible in the traces seen after the introduction of some of the anesthetics (Figure 3 and 4). After this excitement phase, the potentials were diminished or suppressed by the presence of anesthetic.

This effect can be reversed if the anesthetic is washed out with warm air or when a low concentration of anesthetic is used the explant will slowly recover activity with the passage of time even if the anesthetic is not physically washed out. High concentrations of anesthetic will cause permanent suppression of the potentials if the anesthetic is not removed rapidly by the immediate and repeated replacement by warm air. After a dose of anesthetic had been washed out with warm air, the explant

was then susceptible to further doses of the same or other anesthetics.

The increase in frequency of potential production or 'excitement phase' is of interest. It is notable that in spite of the second dose of anesthetic being five times that of the first (Figure 1) the interval between the end of injection and onset of excitation is 80 sec in each case. Possibly this is because the vapor had to penetrate through the same thickness of explant to reach the active focus in each occasion. Unless an inhibitory effect by some of the cells in the explant is present and removed by anesthetic action, this 'excitement phase' must be due to direct stimulation by the anesthetic. This suggests that the excitement stage encountered in humans and animals during induction with anesthetics may also be due to direct stimulation of cortical cells and not to paralysis of inhibitory action.

Zusammenfassung. Die spontanen Potentiale von Telencephalon-Explantaten *in vivo* werden durch anästhetische Gase nach anfänglichem Potentialanstieg vermindert bzw. gehemmt. Die Potentiale kommen in ihrer ursprünglichen Form nach Entfernung des Betäubungsmittels mit warmer Luft wieder zurück, und die Explantate sind dann wieder durch dasselbe oder andere Betäubungsmittel beeinflussbar.

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Tissue Dynamics Laboratory, Pathology Department, University of Texas Medical Branch, Galveston (Texas), May 29, 1961.

The Effect of Puromycin on Protein Metabolism and Cell Division in Fertilized Sea Urchin Eggs¹

It has recently been shown that puromycin is a specific inhibitor of protein metabolism at the S-RNA-ribosome level^{2,3}. Preliminary experiments on the effects of this drug on early sea urchin development indicated that it inhibits the mitotic activity of fertilized eggs in a very characteristic way. When puromycin was added to the eggs some minutes before fertilization at concentrations above 10⁻⁴M, activation and early development proceeded in a seemingly normal way until the 'clear streak' stage, but then development came to a standstill. The 'clear streak' attained a rigid appearance with abnormally sharp borders. The nucleus gradually swelled, but no spindle was observed and no cell divisions occurred. The present report deals with an attempt to correlate this anti-mitotic effect of puromycin with its inhibitory action on

the incorporation of labeled amino acids into protein by the same eggs, *in vivo* and *in vitro*.

Experimental. Puromycin (Lederle Laboratories Division) at varied concentrations was added to unfertilized eggs of the sea urchin *Paracentrotus lividus* (LM), 10 min before fertilization. At various intervals after fertilization, equal egg samples (approximately 10 mg protein) were withdrawn from the egg suspensions. After sedimentation of the eggs, the volume of the medium was reduced to 4 ml, and 75 m μ M of ¹⁴C-L-valine (6.53 mc/mM) was

¹ Supported by a grant from the Swedish Natural Science Research Council. Experiments carried out at the Zoological Station, Naples (Italy).

² M. B. YARMOLINSKY and G. L. DE LA HABA, *Proc. nat. Acad. Sci., Wash.* 45, 1721 (1959).

³ A. VON DER DECKEN and T. HULTIN, *Biochim. biophys. Acta* 45, 139 (1960).

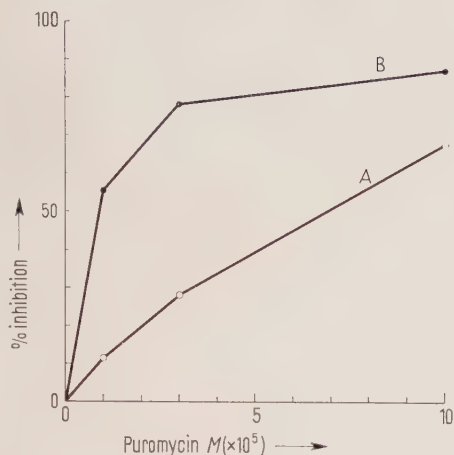


Fig. 1. Inhibition by puromycin of the incorporation of ^{14}C -L-valine into protein by fertilized *Paracentrotus* eggs. A: Intact eggs 35–45 min after fertilization ('clear streak' stage). The eggs (approximately 10 mg protein) were incubated for 10 min (20°C) with $75\text{ m}\mu\text{M}$ ^{14}C -L-valine. Puromycin as indicated was added 10 min before fertilization. B: Mitochondria-free egg homogenate, 40 min after fertilization. Incubation system: 0.75 ml homogenate, $10\text{ }\mu\text{M}$ phosphoenol pyruvate, $1\text{ }\mu\text{M}$ ATP, $0.075\text{ }\mu\text{M}$ ^{14}C -L-valine, and puromycin as indicated. Incubation period 45 min (25°C).

added. The suspension was gently shaken for 10 min (20°C), after which the incorporation was stopped by the addition of trichloroacetic acid. Protein purification and radioactivity determinations were made as described previously⁴. The visible effect of the puromycin treatments on cleavage and development was checked under the microscope on egg samples taken at intervals from the same suspensions.

The effect of puromycin on ^{14}C -L-valine incorporation by cell-free systems was studied in parallel on eggs from the same batch. After fertilization the eggs were freed from jelly by acid treatment⁵, and homogenates were prepared as described previously⁴. The homogenates were centrifuged at $12000 \times g$ for 8 min (0°C), and 0.75 ml of the supernatant was incubated for 45 min at 25°C with $10\text{ }\mu\text{M}$ of phosphoenolpyruvate, $1\text{ }\mu\text{M}$ of ATP, and $0.075\text{ }\mu\text{M}$ of ^{14}C -L-valine in the presence of puromycin at varied concentrations. After incubation, the proteins were isolated and the radioactivity determined as in the *in vivo* experiments.

Results and Discussion. The inhibitory effect of puromycin on amino acid incorporation by fertilized *Paracentrotus* eggs *in vivo* and *in vitro* is illustrated by Figure 1. In the *in vivo* part of this experiment the egg samples, treated with puromycin at the indicated concentrations, were incubated with ^{14}C -L-valine in the interval between 35 and 45 min after fertilization. In 10^{-4} M puromycin 50% of the eggs stopped developing after the 'clear-streak'-stage of the first division, while the rest of the eggs reached the two-cell stage. Development was definitely arrested, however, at the corresponding phase of the second cleavage, when the same typical picture of inhibition was displayed. In spite of the perfectly normal elevation of the fertilization membrane, the two cells became tightly adherent. At the lower puromycin concentrations, a larger portion of the eggs divided, and at a concentration of 10^{-5} M , 40% of the eggs reached the 4-cell stage. The inhibitory effect of puromycin on the incorporation of ^{14}C -L-valine *in vitro* was studied on a

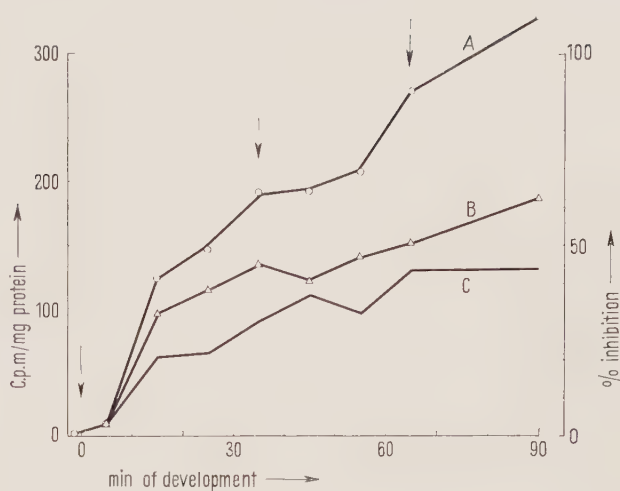


Fig. 2. Effect of puromycin on the velocity of ^{14}C -L-valine incorporation by *Paracentrotus* eggs at different periods of development. A: Eggs in plain sea water. B: Eggs in sea water with 10^{-5} M puromycin. C: % inhibition. Incubation system as in Figure 1 A. Arrows symbolize (from left) Fertilization, 'clear streak', first cleavage in control suspension. Measurements are plotted in the middle of the incubation periods (10 min).

second part of the same egg suspension. The homogenate was prepared 40 min after fertilization.

As is shown by Figure 1, the cell-free incorporation system was considerably more sensitive to puromycin than were the intact eggs. Also with the whole eggs, however, the inhibition became very marked at the higher puromycin concentrations.

The experiment shown in Figure 2 illustrates the gradual development of the puromycin inhibition. In this experiment, the puromycin concentration was 10^{-5} M , and some of the eggs finally reached the 8-cell stage. In the cases, however, when the third cleavage occurred, it was usually unequal and gave rise to 1–4 micromeres.

The mitotic block induced by puromycin is probably a direct effect of the impaired protein metabolism. Special kinds of proteins of importance for the initiation of mitosis may not become produced in sufficient amounts under these conditions. Which functions these proteins have can only be a matter of conjecture. The present data are consistent, however, with the idea that some of them may be related to the formation of the mitotic spindle.

Résumé. La puromycine inhibe fortement l'incorporation de la ^{14}C -L-valine dans les protéines, aussi bien dans les œufs fécondés d'oursins intacts que dans les préparations subcellulaires faites à partir de ceux-ci. Cependant ces préparations se sont montrées nettement plus sensibles à l'action de la puromycine que les œufs entiers. Parallèlement le blocage des divisions cellulaires a été proportionnel à cette action inhibitrice sur le métabolisme des protéines. Le développement s'est arrêté au stade «clear streak».

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The Wenner-Gren Institute, University of Stockholm (Sweden), May 15, 1961.

⁴ T. HULTIN, Exp. Cell Res., in press.

⁵ E. VASSEUR, Acta chem. scand. 2, 900 (1948).

Experimental Production of Very Small Lesions by Electrocoagulation

Lesions in the central nervous system may be made for either of two purposes: (1) for localised *destruction* of nervous tissue (elimination of disordered elements in clinical cases; production of behavioural or histological changes in research work), (2) for *marking* the exact site of the tip of electrodes used in stimulating or recording experiments.

Electrodes used for elimination are generally not very thin, and often have a long non-insulated tip, as fairly large lesions are required. Satisfactory results can then be obtained with alternating currents in the frequency range of a few hundred kc/sec^{1,2}.

In a number of cases, however, the need for thinner electrodes and smaller lesions arises, either because the brain of the experimental animal itself is very small, or because the experiment involves the use of micro-electrodes (as in single unit recording). I found that alternating currents of the type just mentioned produce lesions of poor quality, or even no coagulation at all.

To identify the factor responsible for these unsatisfactory results, I made 'lesions' in the white of hens' eggs, in which the development of the coagulate can be followed under the microscope. It soon became clear that, when the surface area of the non-insulated tip of the electrode is smaller than a certain size, gas bubbles develop in the coagulate at the current strength necessary to produce a lesion. With very small tip sizes, gas bubbles appear first and no coagulate is formed at all.

I have no definite proof as to the nature of this gas. It does not appear to be combustion gas, for no sparks or other evidence of burning are to be seen when it is formed. It is not likely that the gas is water vapour, for the bubbles do not disappear by condensation when the current is switched off. The most plausible hypothesis seems to be that the gas is formed by electrolysis occurring at the electrode tip above a certain current density. Electrolysis, however, can be restricted by the use of higher frequencies.

Therefore, I investigated the influence of the frequency on the quality of lesions made with small electrodes³.

The electrodes used were made of electropolished tungsten wire of various diameters, insulated with glass but for the tip, the surface area of which was established microscopically. The lesions were again made in the white of hens' eggs.

In Figure 1 the results of these experiments are plotted for a range of electrode tip areas against the frequencies used. It is clear that for each frequency there is a minimum electrode tip area with which a good lesion can be made, and that this minimum size shifts to smaller values with increasing frequency. Hence for each electrode size the minimum frequency, below which no lesion of good quality can be produced, is fixed (Figure 2) and the use of thinner electrodes is only possible with higher frequencies.

It may seem, therefore, that the higher the frequency of the generator, the broader its field of application. The difficulties of handling high frequency currents, however, increase with the frequency, because more energy is lost over the capacity of the connecting cable, the electrode insulation, and other stray capacities. As it is important to know the energy delivered at the electrode tip, and exact measurement of this energy is only possible if losses are small in comparison, the best generator for a certain purpose is the one giving a frequency just high enough to produce satisfactory lesions with the electrode size used in the particular experiment.

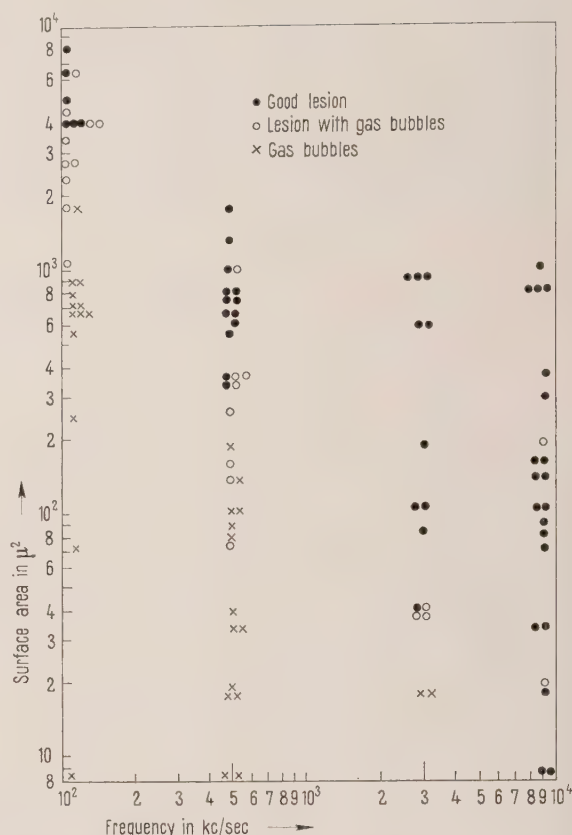


Fig. 1. The quality of a lesion depends on the surface area of the electrode tip and the frequency of the current.



Fig. 2. Tungsten microelectrode used with a lesion current of sufficient frequency, giving a lesion.

Zusammenfassung. Es wird gezeigt, dass Elektrokoagulieren mit kleinen Elektrodenspitzen nur dann möglich ist, wenn hohe Frequenzen gebraucht werden, sonst bilden sich Gasblasen. Je kleiner die Spitze ist, desto höher muss die Frequenz gewählt werden.

C. M. BALLINTJN

Zoological Laboratory of the University of Groningen (Netherlands), June 5, 1961.

¹ O. A. M. Wyss, *Helv. physiol. Acta* 3, 437 (1945).

² R. W. HUNSPERGER and O. A. M. Wyss, *Helv. physiol. Acta* 11, 283 (1953).

³ My thanks are due to the Dr. NEHER, Laboratory of the P.T.T. in Leidsendam (Netherlands), where I enjoyed hospitality to do the first experiments.

Action du LiCl sur de jeunes blastodermes de poulet cultivés *in vitro*¹

L'action spécifique du LiCl sur la morphogénèse est une découverte déjà ancienne. Les malformations qu'il provoque sont connues pour bon nombre d'espèces d'Invertébrés et de Vertébrés anamniotes. En revanche, les quelques travaux publiés à ce sujet sur les Amniotes, à savoir sur les oiseaux, n'ont rien fourni de décisif. LANDAUER² ne constate qu'une augmentation du taux de mortalité parmi ses embryons traités. NAZ et RULON³ injectent du LiCl isotonique dans l'œuf avant son incubation ou après 48 h de développement sans rencontrer de malformations électriques.

La culture *in vitro* m'offre la possibilité de préciser le mode d'action de cet agent sur les oiseaux et d'entreprendre une étude systématique de ses effets. La présente note décrit la technique employée et les premiers résultats de l'étude en cours.

Technique. La culture *in vitro* est celle de NEW⁴, mais légèrement modifiée pour permettre le transport des blastodermes sur différents milieux. Le jaune d'œuf de White-Leghorn est immergé dans une solution de Tyrode isotonique au plasma de poule (HOWARD⁵). Le blastoderme est détaché du vitellus avec une rondelle de membrane vitelline que l'on étale, la face ventrale du blastoderme orientée vers le haut, sur un anneau de verre disposé au fond d'un verre de montre contenant le milieu utilisé. Pour maintenir la tension de la membrane vitelline et faciliter le transport du blastoderme, on emboîte un anneau, d'un diamètre interne légèrement plus grand, sur le premier (GALLERA et NICOLET⁶). Des blastodermes prélevés à des stades précis, depuis la ligne primitive courte jusqu'à l'apparition des premiers somites, sont soumis à l'action de LiCl pendant un laps de temps déterminé (8 h au maximum), puis ils sont transportés sur de l'albumen pur. Ils sont ensuite fixés après 36 à 48 h d'incubation totale.

Le milieu lithiné est composé d'une part d'albumen mélangé à un volume équivalent de Tyrode dans lequel le NaCl a été substitué par le LiCl.

Afin de vérifier si le Tyrode nuit au développement des blastodermes, des témoins sont cultivés pendant un temps comparable sur un milieu à base de Tyrode et d'albumen. La solution saline ne semble pas avoir des effets nocifs, par contre le développement de ces embryons est retardé s'ils sont mis en culture avant la formation du prolongement céphalique. Ces conditions de culture défavorisent donc l'anabolisme protéinique embryonnaire (NEW⁷; BRITT et HERMANN⁸).

Résultats expérimentaux. Le chlorure de lithium agit en premier lieu sur les mouvements morphogénétiques aboutissant à la mise en place des ébauches embryonnaires, secondairement sur leur organisation ultérieure. Les blastodermes sont sensibles au LiCl à tous les stades à partir desquels ils ont été soumis à son action. Les variations individuelles sont importantes, toutefois sans masquer le fait que la sensibilité au lithium diminue brusquement au moment où l'invagination du mésoblaste embryonnaire s'achève et par la suite au début de la neurulation.

Si les blastodermes sont prélevés avant l'apparition du prolongement céphalique et soumis à une action prolongée de lithium, le corps embryonnaire ne se forme pas. L'extension de l'aire vasculaire est entièrement bloquée et l'aire pellucide ne subit qu'une légère elongation. Cependant, du mésoblaste extra-embryonnaire s'est invaginé en formant un croissant postérieur d'îlots sanguins compacts en différenciation.

A partir du stade du prolongement céphalique le lithium ne peut agir que sur l'organisation du corps embryonnaire et la différenciation de ses ébauches. Le cerveau, l'intestin céphalique, le cœur et le tronc manifestent d'évidentes hypomorphoses. Ces embryons ont de nettes tendances à l'omphalocéphalie. Très schématiquement on distingue trois degrés en ordre de gravité décroissante: (1) Le cerveau extrêmement réduit, en particulier l'acrencéphale, constitue un tubule à paroi mince. Il s'engage un peu en profondeur en refoulant l'endoblaste, lequel ne peut pas dans ces conditions former de l'intestin céphalique. La différenciation histologique de ce feuillet est également inhibée. Les ébauches cardiaques rudimentaires occupent leur place primitive des deux côtés du corps. La plaque médullaire reste étalée. Les parties troncales sont sensiblement raccourcies et seulement quelques somites parviennent à s'individualiser. En ce cas, on ne retrouve aucune trace de la ligne primitive en arrière. (2) Quoique la configuration générale de la région céphalique soit comparable, l'état du développement des ébauches est nettement meilleur. Malgré l'absence de l'intestin céphalique, la cytodifférenciation de l'endoblaste est normale. Les ébauches cardiaques paires parviennent à se différencier et souvent se rapprochent de la ligne médiane et se placent au-dessus du rhombencéphale comme chez les omphalocéphales typiques. Le tube nerveux est déjà plus typique, la chorde dorsale est bien différenciée et le nombre de somites normal. Toutes ces formations convergent subitement en une masse cellulaire apparemment homogène qui rappelle le bourgeon tronco-caudal. (3) L'intestin céphalique n'est fermé qu'en avant du cerveau, plus en arrière l'endoblaste, correspondant à la voûte de l'intestin, est repoussé vers le bas par les vésicules cérébrales dont les parois sont plissées irrégulièrement. La seule anomalie importante du tronc est que les rangées somitiques s'accroissent parfois en arrière et donnent naissance à une lignée de somites médians sous la chorde. Rarement, celle-ci s'intercale entre la chorde et le tube neural. La chorde est raccourcie et trop massive et se termine à ce niveau par un épaississement en massue, tandis que les somites médians ne forment bientôt plus qu'une sangle somitique sous le plancher de la moelle.

La topogénèse des embryons traités à la neurulation n'est presque plus atteinte par l'action du LiCl. Tout au plus, le cœur peut prendre une forme atypique et occasionnellement on retrouve des somites médians postérieurs. Seul le cerveau, en particulier l'acrencéphale, est encore réellement réceptif au LiCl. Ses atteintes sont de types disparates. Elles se traduisent soit par des irrégularités de la forme de l'encéphale, soit par sa réduction quantitative. Les ébauches oculaires généralement mal conformées sont dans ce dernier cas toujours absentes.

Conclusions. La sensibilité au LiCl diminue au cours du développement. Aux stades jeunes, le LiCl empêche la formation des parties axiales. A considérer la gravité des effets, ce cas est comparable à celui des embryons lithinés amorphes de *Rana fusca* que PASTEELS⁹ a signalés. L'ébauche neurale est la plus touchée et toujours la première à présenter des malformations. Cette extrême susceptibilité s'explique probablement par l'ampleur des

¹ Travail subventionné par le Fonds national suisse de la Recherche scientifique.

² W. LANDAUER, Poultry Sci. 8, 301 (1929).

³ J. F. NAZ et O. RULON, Anat. Rec. 96, 555 (1946).

⁴ D. A. T. NEW, J. Embryol. exp. Morph. 3, 326 (1955).

⁵ E. J. HOWARD, J. Cell. comp. Physiol. 41, 237 (1953).

⁶ J. GALLERA et G. NICOLET, Exper. 17, 134 (1961).

⁷ D. A. T. NEW, J. Embryol. exp. Morph. 7, 146 (1959).

⁸ L. G. BRITT et H. HERMANN, J. Embryol. exp. Morph. 7, 66 (1959).

⁹ P. PASTEELS, J. Embryol. exp. Morph. 1, 1 (1952).

événements morphogénétiques et histogénétiques dont le cerveau est le siège durant cette période du développement. Du mésoblaste axial, le matériel chordal est le premier à s'individualiser, contrairement à ce qu'on observe chez les amphibiens. La conversion du matériel chordal en somites observée chez les amphibiens (LEHMANN¹⁰; PASTEELS⁹) ne se rencontre jamais chez le poulet.

Corrélativement à l'importance de l'atteinte, l'expansion du mésoblaste extra-embryonnaire, qui engendre l'aire vasculaire, est plus ou moins bloquée en cours de progression.

Summary. The young blastoderms cultivated *in vitro*, by the method of NEW, slightly modified, grow ab-

normally on a medium of albumen mixed with LiCl solution. It acts in the first place upon the morphogenesis, secondarily upon the differentiation of organs, if they are able to develop.

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Laboratoire d'Embryologie expérimentale à l'Institut d'Anatomie de l'Université de Genève (Suisse), le 2 mai 1961.

⁹ J. PASTEELS, Arch. Biol. 56, 105 (1945).

¹⁰ F. E. LEHMANN, Roux Arch. Entw. Mech. 136, 112 (1937).

Cytochrome Oxidase Activity in Skeletal Muscle and the Diaphragm of Intermittently Starving Rats

Previous findings from our laboratory revealed that, in rats adapted to intermittent starvation, there are a number of functional and morphological changes¹. The energy metabolism of these animals is characterized by an increase in $\dot{Q}O_2$ of tissues² and in the basal oxygen consumption *in vivo*³. We investigated, therefore, whether these changes are reflected in the activity of the terminal respiratory enzyme—cytochrome oxidase—particularly in skeletal muscle.

The enzyme activity was estimated manometrically, using the method described by SCHNEIDER and POTTER⁴ at a temperature of 39°C. In experiments with skeletal muscle, the rat was sacrificed, skinned and, after evisceration, the fat was mechanically separated. The muscles and skeleton were homogenised in distilled water (1:9) by the procedure described by JÁNSKÝ⁵ in an ice-cooled Turmix-homogenizer and aliquot portions in a Potter-Elvehjem homogenizer. The skeleton was, according to preliminary experiments, a minimal cytochrome oxidase activity. To the diaphragm distilled water was added, one hundred times its weight. In the homogenates, the nitrogen was estimated by the Microkjeldahl method. All animals were included in the experiment after 24 h fasting; before fasting the animals received a weighed ration.

Male rats, Wistar strain, were used. Some of the animals were adapted to intermittent starvation as follows: during the first two weeks, they were fed on alternate days; during the subsequent two weeks, three times a week, and for the remainder of the experiment (2–6 weeks) only twice a week. The total caloric intake of these animals was by about 30–40% lower than in *ad libitum* fed controls. Another group was fed daily, for a period of 6–8 weeks, a reduced ration, corresponding to ca. 60% of the caloric intake of the control animals. All groups were fed a standard diet, containing 50% of the caloric volume of carbohydrate, 25% fat and 25% protein⁶.

As demonstrated in the Table, the cytochrome oxidase activity (when calculated in relation to the nitrogen content of the tissue) is significantly higher in intermittently starving animals, both in skeletal muscle and the diaphragm. In rats fed daily a reduced ration, there is practically no change, as compared with the control group.

Among other consequences, intermittent starvation leads to an overall increase of metabolism. As has been shown in a previous paper³, this increase is probably mainly due to the higher nutrient supply during periods of food intake when intermittently starving rats develop

considerable hyperphagia. The greater demands on the metabolism resulting from the intermittent increase of nutrient supplies probably lead to the establishment of a higher metabolic level. Finally a change of the enzyme systems involved, in this case cytochrome oxidase, develops. The results of experiments with rats subjected to simple chronic caloric undernutrition support this assumption. These animals have practically the same food intake as rats adapted to intermittent starvation but no changes in the $\dot{Q}O_2$ or in the cytochrome oxidase activity can be detected in these animals.

The observed increase of cytochrome oxidase activity in rats accustomed to intermittent starvation is interesting, as it is known that levels of this enzyme decrease with age⁷. Thus, the elevated values found in our experiments, similarly as other findings in rats adapted to this nutritional pattern (growth activity of explanted muscle and liver⁸, higher $\dot{Q}O_2$ levels of tissues², increased basal oxygen consumption *in vivo*³) correspond to a lower biological age of the animal.

Cytochrome oxidase activity in skeletal muscle and diaphragm of intermittently starving rats (in $\mu\text{l } O_2/\text{mg N/h}$)

Group	Number of animals	Weight g	Skeletal muscles and skeleton	Diaphragm
Controls	12	204.5 ± 7.9	65.01 ± 14.14	759.50 ± 77.59
Intermittent starvation	6	128 ± 9.4	144.95 ± 28.45 ^b	1304.53 ± 153.71 ^a
Simple under-nutrition	6	110 ± 7.1	81.10 ± 16.3	828.92 ± 87.14

The figures are averages ± S.E. ^a The difference of values as compared with controls is statistically significant for $P < 0.01$. ^b for $P = 0.02$.

¹ P. FÁBRY, Physiol. bohemoslov. 4, 33 (1955). – P. FÁBRY and V. KUJALOVÁ, Naturwissenschaften 45, 373 (1959). – R. PETRÁSEK, Nature (Lond.) 183, 323 (1959). – E. HOLEČKOVÁ and P. FÁBRY, Brit. J. Nutr. 13, 260 (1959). – P. FÁBRY, V. KUJALOVÁ, and R. PETRÁSEK, Nahrung 3, 642 (1959).

² R. PETRÁSEK, Čs. fysiologie 9, 453 (1960).

³ P. FÁBRY, R. PETRÁSEK, and L. KRULICH, Physiol. bohemoslov., in press (1961).

⁴ W. C. SCHNEIDER and V. R. POTTER, J. biol. Chem. 149, 217 (1943).

⁵ L. JÁNSKÝ, Acta Soc. zool. Bohemoslov. 21 (2), 164 (1957).

⁶ P. FÁBRY, Čs. fysiologie 8, 529 (1959).

⁷ H. O. KUNKEL and J. E. CAMPBELL, J. biol. Chem. 198, 229 (1952).

⁸ E. HOLEČKOVÁ, O. POUPA, and P. FÁBRY, Physiol. bohemoslov. 8, 15 (1959).

Zusammenfassung. Es wurde die Aktivität der Cytochromoxydase von Skelettmuskulatur und Zwerchfell intermittierend hungernder Ratten und bei chronisch durch verminderte Tagesrationen unterernährten Ratten untersucht. Bei den an intermittierendes Hungern adaptierten Ratten war die auf den Gewebestickstoff bezogene Aktivität dieses Enzyms um 80–100% erhöht im Vergleich zu den *ad libitum* gefütterten Kontrolltieren. Bei

den einer gewöhnlichen kalorischen Unterernährung ausgesetzten Tieren war die Cytochromoxydaseaktivität praktisch dieselbe wie die der Kontrolltiere.

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Die Wirkung von Persantin auf Herzmuskel-Sarkosomen

Bei der klinischen und experimentellen Anwendung einer neuen coronargefässerweiternden Verbindung, dem 2,6-Bis-(diäthanolamino)-4,8-dipiperidino-pyrimido-(5,4-d)-pyrimidin, Persantin®, wurden Ergebnisse erhalten, die für einen zusätzlichen Stoffwechseleffekt dieser Substanz sprechen. Neben einer echten Leistungssteigerung des Myocards^{1,2} konnte bei dekompensierten Herzinsuffizienzen unter der Persantintherapie ein schnellerer Rückgang der Transaminase-Aktivitäten des Serums sowie ein schnelleres Absinken der erhöhten Milchsäure- und Brenztraubensäure-Spiegel im Blut beobachtet werden^{3,4}. Es wurde ein direkter Eingriff in energieliefernde Stoffwechselprozesse vermutet, da beim Hund nach hypoxämischer Schädigung des Herzens die erniedrigten ATP-Werte der Herzmuskulatur unter Persantin deutlich anstiegen⁵. Persantin inhibiert nach SIESS den Erschöpfungs- und Energiemangelstillstand des Rattenvorhofs, eine Beobachtung, welche von ihm auf Stoffwechsel-Effekte zurückgeführt wird⁶.

Im Stoffwechsel erfolgt der Aufbau energiereicher Phosphatverbindungen vom Typ der ATP hauptsächlich durch oxydative Phosphorylierung in den Mitochondrien. Wir untersuchten deshalb den Einfluss verschiedener Konzentrationen von Persantin auf die oxydative Phosphorylierung von Herzmuskel-Mitochondrien (Sarkosomen) *in vitro*.

Die Sarkosomen wurden aus frischen Rinderherzen nach einer früher beschriebenen Methode isoliert; gleiches gilt auch für die Messung der P/O-Quotienten mit Succinat, β -Hydroxybutyrat und Ketoglutarat/Malonat im Warburg-Versuch^{7,8}. Insgesamt wurden 130 Manometergefäß-Versuche durchgeführt. Neben frisch isolierten und morphologisch wie funktionell intakten Sarkosomen wurden vergleichsweise auch künstlich geschädigte Sarkosomen in die Versuche eingesetzt. Die Schädigung erfolgte durch Lyophilisierung und Resuspendierung frischer Sarkosomenfraktionen. Dadurch entstanden strukturelle Schäden der Partikel (Quellungen, partieller Zerfall) mit entsprechenden Funktionsausfällen (Senkung der P/O-Quotienten). Ähnliche morphologische Schäden und Funktionsausfälle bestehen auch *in vivo* bei Sarkosomen im hypoxämischen Myocard^{9–11}. Eine eingehende Beschreibung der Versuche erfolgt an anderer Stelle.

Ergebnisse. Wie die Tabelle I zeigt, wurde durch steigende Konzentrationen von Persantin bei frisch isolierten Sarkosomen die Sauerstoffaufnahme mit Succinat als Substrat zunächst stärker gesenkt als die Phosphataufnahme. Dadurch kam es zu einem geringen, statistisch jedoch nicht gesicherten Anstieg der P/O-Quotienten. Bei β -Hydroxybutyrat und Ketoglutarat/Malonat als Substrate war die Senkung der Sauerstoffaufnahmen unter Persantin sowohl bei Sarkosomen als auch bei Lebermitochondrien deutlich stärker und führte sogar zu einer *signifikanten* Steigerung der P/O-Quotienten (Tabelle II).

Konzentrationen ab 10^{-4} m Persantin/Ansatz hemmten dann bei allen Substraten die Phosphorylierung signifikant und zunehmend stärker als die Atmung.

Völlig anders verhielten sich geschädigte Sarkosomen unter Persantin: bei unveränderter Atmung wurde schon durch 1×10^{-6} bis 5×10^{-6} m Persantin/Ansatz die Phosphataufnahme gesteigert, die P/O-Quotienten stiegen um

Tab. I. Phosphat- und Sauerstoffaufnahmen von Rinderherz-Sarkosomen mit Succinat (0,005 m) als Substrat unter Persantin. 42 Versuche (24/18). Sarkosomen: 0,5 bzw. 0,8 mg N/Ansatz. Aufnahmen in μ g-Atomen. Die mit einem * bezeichneten P/O-Quotienten sind gegenüber der Kontrolle signifikant erhöht.

Molarität von Persantin im Ansatz	Frisch isoliert			Geschädigt (Lyophilisat)		
	P	O	P/O	P	O	P/O
Kontrolle	3,74	2,14	1,75	1,54	1,50	1,03
1×10^{-6}	3,78	2,10	1,80	2,84	1,50	1,90*
5×10^{-6}	3,67	1,97	1,86	2,84	1,50	1,90*
1×10^{-5}	3,60	1,90	1,89	2,32	1,50	1,55
1×10^{-4}	2,88	1,63	1,77	0,40	1,31	0,31
1×10^{-3}	1,61	1,22	1,32	0,00	0,54	0,00

Tab. II. Beeinflussung der Phosphat- und Sauerstoffaufnahmen frisch isolierter Rinderherzsarkosomen durch Persantin bei Ketoglutarat/Malonat (je 0,01 m) und β -Hydroxybutyrat (0,01 m) als Substrate. 24 Versuche. Sarkosomen-N/Ansatz 0,68 mg bzw. 0,5 mg.

Molarität von Persantin im Ansatz	Ketoglutarat/Malonat			β -Hydroxybutyrat		
	P	O	P/O	P	O	P/O
Kontrolle	4,20	1,28	3,28	2,41	1,04	2,32
1×10^{-6}	4,22	1,26	3,35	2,45	1,06	2,31
5×10^{-6}	4,04	1,18	3,42	2,40	0,99	2,42
1×10^{-5}	4,00	1,15	3,48	2,50	0,94	2,66*
1×10^{-4}	3,82	1,01	3,78*	2,22	0,78	2,85*
1×10^{-3}	1,89	0,74	2,55	1,20	0,62	1,94

¹ H. W. KNIPPING, W. BOLT und K. MIKULICZ, *Arzneimittelforsch.* 10, 364 (1960).

² L. DÜTSCH, *Ther. Gegenw.* 99, 228 (1960).

³ E. ALBACH, *Med. Welt* 1960, 993.

⁴ S. GREIF, *Wien. med. Wschr.* 110, 463 (1960).

⁵ TH. HOCKERTS und G. BÖGELMANN, *Arzneimittelforsch.* 9, 47 (1959).

⁶ M. SIESS, 27. Jahrestagung Deutsche Gesellschaft für Kreislauf-Forschung, Bad Nauheim (1961).

⁷ G. LAUDAHN, *Z. phys. Chem.* 314, 95 (1959); 318, 186 (1960).

⁸ G. LAUDAHN und C. J. LÜDERS, *Arzneimittelforsch.* 10, 978 (1960).

⁹ F. BÜCHNER, *Schweiz. med. Wschr.* 88, 73 (1958).

¹⁰ E. MOELBERT und D. GUERRITORE, *Beitr. path. Anat.* 117, 32 (1957).

¹¹ E. MOELBERT, *Beitr. path. Anat.* 118, 203 (1957).

rund 84% an. Gleichartig war dagegen wieder die Hemmung der Phosphat- und Sauerstoffaufnahmen durch 10^{-4} m Persantin/Ansatz; höhere Konzentrationen führten dann zu einer völligen Entkopplung von Atmung und Phosphorylierung. Alle Versuchsergebnisse waren mit einer Schwankungsbreite der Messwerte von $\pm 8\%$ gut reproduzierbar.

Die Befunde sprechen dafür, dass Persantin bei geschädigten Sarkosomen eine bessere Sauerstoffutilisation bewirken kann, das heisst zu einer festeren Kopplung von Atmung und Phosphorylierung führt. Bei gleichbleibender Sauerstoffaufnahme steigende P/O-Quotienten bedeuten eine eindeutige Steigerung der ATP-Synthese.

Damit besteht eine deutliche Korrelation zu den Versuchsergebnissen *in vivo*, bei denen eine Zunahme der ATP-Konzentration im geschädigten Myocard unter der Persantintherapie nachgewiesen wurde⁵.

Zusammenfassend ergibt sich, dass die direkte und günstige Beeinflussung der Sarkosomenfunktion durch Persantin *in vitro* möglicherweise auch die Ursache für die *in vivo* beobachtete Wirkung dieser Substanz auf den Herzstoffwechsel ist.

Summary. Addition of Persantin to respiring beef heart mitochondria leads to a considerable increase of the P/O-ratios in the presence of various substrates. Apparently, Persantin is capable of bringing about a tighter coupling of oxidation and phosphorylation.

G. LAUDAHN

Hauptlabor des Städtischen Krankenhauses Berlin-Wilmersdorf, Berlin (Deutschland), 12. Juni 1961.

Étude sur le choc produit par le polysaccharide du bacille du colon chez le rat porteur de tumeur (Note préliminaire)

Plusieurs auteurs^{1,2} ont déjà signalé assez indépendamment l'observation selon laquelle la présence d'une tumeur augmentait singulièrement la mortalité chez des animaux qui recevaient une certaine quantité de polysaccharide bactérien. Des travaux, comme ceux de JONES et HOWELL³, renforcent l'idée d'une sélectivité d'action.

Nous avons utilisé pour cette étude deux vieilles préparations, l'une domestique et l'autre commerciale, de polysaccharides provenant de l'*Escherichia coli*. Ces produits injectés à hautes doses chez la rate porteuse de tumeur provoquaient un choc particulièrement violent et l'animal mourait en 4 à 8 h, tandis que chez des rates ne portant pas de tumeur, des doses beaucoup plus fortes étaient très bien tolérées.

Nos expériences étaient effectuées chez des rates de souche Sprague-Dawley, de 200 à 300 g, porteuses ou non de tumeur (e.g. lymphosarcome de Murphy-Sturm). Les tumeurs employées avaient été repiquées plusieurs dizaines de fois chez des rates de cette souche et ne régresaient jamais spontanément. Dès que la tumeur dépassait la grosseur d'environ 2 cm de diamètre, une injection unique de polysaccharide d'*E. coli* (e.g. souche 0127, Difco, Détroit), à la dose d'une fraction de mg à 10 mg intra-péritonéale, ou à la dose de 10 à 100 mg sous-cutanée, devenait rapidement très meurtrière et les animaux succombaient au cours d'une forme de syndrome d'alarme dont quelques auteurs ont déjà parlé et qui ressemble à celui que provoque une très forte dose de polysaccharide chez un animal sans tumeur. DONNELLY et HAVAS⁴ ont déjà d'ailleurs entrepris une étude histologique en rapport plus spécialement avec ce syndrome. Les injections intra-tumorales se montraient encore plus sélectives, mais nous ne les avons utilisées que pour vérifier l'effet des médicaments adjuvants dont nous parlons plus loin.

Si la tumeur était trop petite, l'animal entraînait en «stress» aigu au bout de 4 à 6 h, mais dans les heures suivantes ces symptômes disparaissaient et l'animal se rétablissait rapidement.

Chez les rats qui ne portaient pas de tumeur, on pouvait évidemment provoquer un choc violent avec de fortes injections intra-péritonéales, mais nous n'avons jamais tué ces animaux avec des injections sous-cutanées.

Nous avons cherché à définir quelques modalités dans l'apparition de ce choc, surtout en associant d'autres médicaments susceptibles d'en modifier l'aspect. C'est ainsi que nous avons vu que certaines médications pré-administrées (comme l'hyposulfite de soude, la dibenzylène et l'acide ascorbique), aggravaient le syndrome, tandis que d'autres (comme le chlorure de magnésium, le salicylamide et le composé «S» de REICHSTEIN) pouvaient retarder plus ou moins l'*exitus*. L'examen post-mortem chez les animaux ainsi traités montrait des lésions variées: outre la liquéfaction ou la nécrose hémorragique du centre de la tumeur qui était constante, même sans ces dernières médications, on observait fréquemment un œdème tissulaire généralisé et présence d'une urine très pigmentée dans la vessie, et plus rarement une diarrhée également pigmentée, des surrénales atrophiques, une distension intestinale exagérée et même de la nécrose aiguë du foie.

En général, les hormones stéroïdes que nous avons administrées, jusqu'à des doses de quelques milligrammes, 1 h avant le polysaccharide, malgré leur diversité reconnue d'action (e.g. cortisone, désoxycorticostérone, estradiol, déhydroépiandrostérone, Presuren⁵) et sauf le composé «S» mentionné plus haut, apportaient une protection efficace contre la léthalité et souvent contre le choc. On a déjà partiellement investigué cet effet de protection chez des animaux sans tumeur⁶.

Certaines autres hormones comme l'ACTH, la GTH et la STH, pouvaient protéger de la léthalité, mais le choc général restait souvent violent. Dans le cas des hormones stéroïdes, l'action du polysaccharide au niveau de la tumeur était manifestement atténuée, ce que beaucoup d'auteurs ont déjà noté, du moins avec les glucocorticoïdes^{2,7}. On peut facilement vérifier cette action en injectant le polysaccharide dans la tumeur. Dans le cas du

¹ M. J. SHEAR, J. Nat. Cancer Inst. 4, 461 (1943–1944). – P. A. ZAHL, J. Nat. Cancer Inst. 11, 279 (1950–1951). – H. F. HAVAS, A. J. DONNELLY et S. I. LEVINE, Cancer Res. 20, 393 (1960).

² H. TAUBER et W. GARSON, Proc. Soc. exp. Biol. Med. 97, 886 (1958).

³ R. S. JONES et E. V. HOWELL, Amer. J. Pathol. 35, 704 (1959).

⁴ A. J. DONNELLY, H. F. HAVAS et M. E. GROESBECK, Cancer Res. 18, 149 (1958).

⁵ Presuren: 21-OH prégnane 3,20-dione, Schering AG, Berlin-Ouest.

⁶ H. J. BEIN et R. JAKES, Exper. 16, 24 (1960).

⁷ S. N. PRADHAN, B. ACHINSTEIN et M. J. SHEAR, Cancer Res. 16, 1062 (1956). – I. C. DILLER, B. BLAUCH et L. V. BECK, Cancer Res. 8, 591 (1948).

lymphosarcome de Murphy-Sturm, cette tumeur finissait toujours par disparaître après traitement intense au polysaccharide, mais cela se produisait de façon différente suivant l'hormone injectée. Il faut dire que la tumeur dont nous nous servions régressait souvent sous l'effet des médications adjuvantes seules.

Des expériences ont été effectuées chez des rates portant la tumeur de Walker 256. Dans cette série le choc initial pouvait être moins marqué, mais les rates survivaient assez facilement, à moins que les tumeurs soient beaucoup plus grosses ou qu'on ait ajouté des médications qui accentuaient le stress. Dans aucun cas d'ailleurs, cette dernière tumeur n'a régressé complètement, même si certaines médications causaient une involution initiale prononcée. On sait déjà l'action préférentielle des polysaccharides au niveau des sarcomes.

Les polysaccharides que nous utilisions avaient été isolés deux ans auparavant et gardés ensuite à la température du laboratoire, et comme on a déjà remarqué⁸ que le pouvoir actif anti-tumoral diminuait avec le temps dans ces préparations, cela peut expliquer pourquoi nous devions utiliser des doses beaucoup plus fortes et moins critiques que celles qu'on emploie généralement. Nous avons remarqué d'ailleurs que nos préparations une fois dissoutes (nous dissolvions la poudre de polysaccharide

dans une quantité minime d'eau distillée), perdaient rapidement après quelques jours tout pouvoir de provoquer un choc chez l'animal cancéreux.

Dans les mêmes circonstances expérimentales, nous avons aussi utilisé à hautes doses une préparation de polysaccharide, également vieille de deux ans, extraite de culture de *Serratia marcescens*; celle-ci ne provoquait pas de choc initial et la mort des animaux, si elle survenait, n'apparaissait que tardivement au bout de 24 à 72 h, due probablement aux métabolites provenant de la tumeur nécrosée.

Summary. Aged preparations of polysaccharide from *E. coli* were highly effective in inducing a lethal shock in rats bearing a tumor. This effect may be increased by some medications, while various steroid hormones afforded an efficient protection.

P. LINDSAY

Faculté de Médecine, Université Laval, Québec (Canada), le 21 avril 1961.

⁸ H. J. CREECH, L. H. KOEHLER, H. F. HAVAS, R. M. PECK et J. ANDRE, *Cancer Res.* 14, 817 (1954).

Isolation from Rat Brain of a Metabolic Product, Desmethylimipramine, that Mediates the Anti-depressant Activity of Imipramine (Tofranil)¹

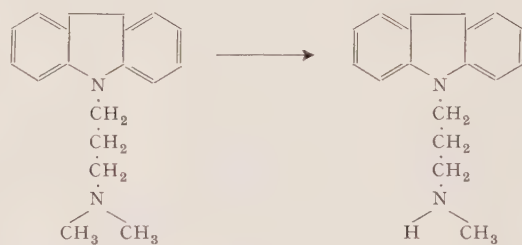
The effectiveness of imipramine (Tofranil) in the treatment of endogenous depression, first reported by KUHN², has now been confirmed by numerous clinical studies. The drug exerts weak chlorpromazine-like effects in normal animals and man, while it exerts an antidepressant action in depressed patients only after repeated doses over a period of several weeks. Recent studies from this laboratory have shown that imipramine counteracts the syndrome of central effects elicited in rats by reserpine and by a synthetic benzoquinolizine, without inhibiting the actions of chlorpromazine³. Again, there is a delay in onset of action, several hours elapsing before this action appears. These considerations suggested to us that both the anti-reserpine and the clinical antidepressant effects of imipramine might be mediated through a metabolic product.

The present communication describes the isolation and identification from brain of a metabolite of imipramine which appears to be responsible for the antidepressant action of the drug. The metabolite, desmethylimipramine (DMI), is derived from imipramine *in vivo* by the removal of one methyl group from the sidechain nitrogen as follows:

The antidepressant activity of imipramine was determined in male Sprague Dawley rats (160-180 g) by the prevention or reversal of the reserpine-like syndrome⁴ elicited by the intraperitoneal administration of 10 mg/kg of RO-4-1284⁵, or the intravenous injection of 2 mg/kg of reserpine. The concentration of imipramine in brain was assayed by a fluorometric procedure⁶.

Brain levels of imipramine were measured in rats after the intraperitoneal injection of 40 mg of imipramine per kg. The drug readily entered the brain and in 45 min had attained a level of 14 µg/g; the level then declined rapidly and in 3 h was less than 3 µg/g. If RO-4-1284 was injected 45 min after imipramine, the latter compound had no obvious effect on the reserpine-like syndrome elicited by

the benzoquinolizine. However, if RO-4-1284 was given 3 h after imipramine, the central effects of the benzoquinolizine were now prevented even though the imipramine level was less than 3 µg/g of brain. From these results it is clear that the anti-reserpine activity of imipramine was not directly related to its concentration in brain.



Imipramine

Desmethylimipramine (DMI)

¹ Presented in part as an abstract in *Fed. Proc.* 20, 321 (1961). Some of the material in this communication is taken from a dissertation by J. V. DINGELL to be submitted to the Graduate School of Georgetown University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

² R. KUHN, *Schweiz. med. Wschr.* 87, 1135 (1957).

³ F. SULSER, J. WATTS, and B. B. BRODIE, *N. Y. Acad. Sci.*, in press.

⁴ Rats treated with RO-4-1284 (10 mg/kg i.p.) or reserpine (2 mg/kg i.v.) exhibit blepharospasm and an almost complete lack of spontaneous motor activity, remaining motionless in a peculiar hunched-back position. In addition they show little response to stimuli such as sound or touch. In imipramine pretreated animals, these signs are prevented or reversed. Reversal is evidenced by a pronounced and persistent hyperactivity of a 'compulsory' exploratory nature.

⁵ The reserpine-like properties of synthetic benzoquinolizines were first described by A. PLETSCHER et al., *Arch. exp. Path. Pharmacol.* 232, 499 (1958). The chemical structure of RO-4-1284 is 2-hydroxy-2-ethyl-3-isobutyl-9,10-dimethoxy-1,2,3,4,6,7-hexahydrobenzo(a)quinolizine. This compound was generously donated by Hoffmann-La Roche Inc.

⁶ J. R. GILLETTE, J. V. DINGELL, and G. P. QUINN, *Fed. Proc.* 19, 137 (1960).

The presence of considerable amounts of a metabolic product in brain was established as follows: Pooled brains from five animals given imipramine 3 h previously were homogenized with three volumes of water, the homogenate was alkalized with 1.0 N NaOH to about pH 12, and shaken with three volumes of heptane containing 1.5% isoamyl alcohol. Phenolic material was removed by shaking the heptane phase with a small volume of 0.1 N NaOH. Basic material in the heptane phase was returned to an aqueous phase by shaking with $\frac{1}{3}$ volume of 0.2 M phosphate buffer, pH 5.9, which would extract only a small fraction of any imipramine that might be present. Traces of imipramine in the buffer phase were removed by shaking with three volumes of the heptane-isoamyl alcohol mixture, and the buffer phase was then alkalized with 3 N NaOH. The fluorescence spectra of this solution, examined with a spectrofluorometer, revealed material with fluorescent characteristics similar to those of imipramine (activation maximum: 295 m μ , fluorescence maximum: 415 m μ , uncorrected). The results indicated that a heptane-soluble metabolite had accumulated in brain in amounts equivalent to about 10 μ g of imipramine per g of tissue. The material was then extracted into heptane.

The metabolite was identified as desmethylinipramine (DMI) as follows:

Gas Chromatography. An aliquot of the heptane phase was evaporated to dryness under reduced pressure, and the residue dissolved in a small amount of acetone. The resulting solution was dried over MgSO₄ and evaporated to about 0.1 ml at room temperature under a stream of nitrogen. The material was subjected to gas chromatography at 175°C using a column of 0.75% Silicone Polymer, SE 30 Gas-Chrome P (mesh-size 100–140), and an argon pressure of one atmosphere⁷. The retention time of the principal component was identical to that of DMI, the monomethyl analogue of imipramine, a metabolite previously isolated by HERRMANN and PULVER⁸ from urine, lung, and liver of animals given imipramine.

Paper Chromatography. Another aliquot of the heptane phase was evaporated to dryness under reduced pressure and the residue dissolved in a small volume of methanol. The methanol solution was applied to Whatman 3 paper, impregnated with peanut oil⁹. The chromatograms were developed by ascending chromatography with a solvent system consisting of concentrated ammonia and methanol (1:1). The chromatogram was irradiated with a shortwave ultraviolet lamp (Model SL 2537 Mineralight lamp). No fluorescent material was seen at room temperature, but on cooling the paper with liquid nitrogen, a brilliant fluorescent spot appeared with an R_f value (0.23) corresponding to DMI. No fluorescent spot was found having an R_f value (0.33) corresponding to desdimethyl-

imipramine, the primary amine analogue of imipramine. When the chromatogram was sprayed with diazotized *p*-nitroaniline and HCl as described by HERRMANN et al.⁹ the fluorescent material formed a blue spot similar to that formed from authentic DMI.

The accumulation of DMI in brain after administration of imipramine suggested that the action of the latter drug might be mediated through the metabolic product. Further experimental support of this was provided by giving imipramine in repeated doses, 20 mg/kg, every 6 h over a period of 24 h. 4 h after the last dose, the animals were given either RO-4-1284 or reserpine. No sedation occurred, and in about 1 h the animals displayed marked hyperactivity lasting over several hours. Analysis of brain showed negligible amounts of imipramine and about 20 to 30 μ g of DMI per g of tissue.

Pharmacological studies have shown that DMI is a new type of antidepressant¹⁰. It does not elicit excitation in the normal rat nor does it block monoamine oxidase or affect brain amines. The antidepressant action of DMI becomes dramatically evident in studies which show that in rats pretreated with the drug the effects of RO-4-1284 or reserpine are reversed. The action of the drug in this regard is far more rapid and potent than that of imipramine. In fact imipramine itself does not seem to have this action for it actually inhibits the action of DMI. Preliminary clinical studies in collaboration with N. KLINE (unpublished) indicate that DMI is potent and rapidly acting in endogenous depression.

Zusammenfassung. Nach Verabreichung von Imipramin (Tofranil) wurde aus dem Gehirn von Ratten Desmethylinipramin, das N-Monomethylderivat, isoliert. Dieser Metabolit beziehungsweise dessen Anreicherung *in vivo* scheint für die antidepressive Wirkung von Imipramin verantwortlich zu sein.

J. R. GILLETTE, J. V. DINGELL, F. SULSER, R. KUNTZMAN, and B. B. BRODIE

Laboratory of Chemical Pharmacology, National Heart Institute, National Institutes of Health, Bethesda (Maryland), July 17, 1961.

⁷ We thank Dr. W. J. A. VAN DEN HEUVEL for performing the gas chromatography analyses.

⁸ B. HERRMANN and R. PULVER, Arch. int. Pharmacodyn. 126, 454 (1960). Reference samples of desmethylinipramine and desdimethylipramine were obtained through the courtesy of Dr. F. HAEFLIGER, J. R. Geigy, S.A., Basel.

⁹ B. HERRMANN, W. SCHINDLER, and R. PULVER, Med. exp. 1, 381 (1959).

¹⁰ F. SULSER, J. V. DINGELL, J. R. GILLETTE, and B. B. BRODIE, in preparation.

Biophysikalische Studien mit synthetischem Lecithin als Weg zu neuartigen Chemotherapeutika

In unserer letzten Mitteilung¹ haben wir dargelegt, dass dem Lecithin vermutlich die biologische Funktion eines «carriers» beim Ionentransport durch den lipoiden Teil der Zellwand zukommt.

In einem einfachen biophysikalischen Modell konnten wir zeigen, dass Lecithin, gelöst in CCl₄, befähigt ist, Anionen aus wässriger Lösung in die lipide Phase überzuführen, wenn in der wässrigen Phase zugleich gewisse Kationen vorhanden sind. Aus messtechnischen Gründen

wurde als Anion Tropäolin verwendet. Die Bindungsfähigkeit zwischen Lecithin und diesem Farbstoff ist stark abhängig von der Art und Konzentration der anwesenden Kationen. Es wurde gefunden, dass insbesondere polybasische Verbindungen wie Protamin und Polymyxin den Transport in die lipide Phase fördern.

In weiteren Versuchen stellten wir fest, dass in CCl₄ gelöstes Lecithin befähigt ist, in Wasser gelöstes Trypaflavin (Kation) in die lipide Phase überzuführen und dass dieselben Kationen, die den Übergang von Tropäolin fördern,

¹ R. HIRT und R. BERCHTOLD, Med. exp. 2, 269 (1960).

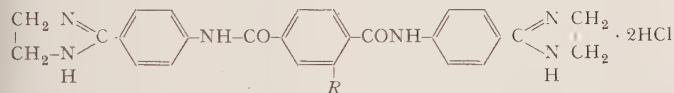
hier nun hemmen. Auch hier sind Protamin und Polymyxin besonders aktiv. Figur 1 und 2 zeigen als Vergleich die Absorption von Trypaflavin bzw. Tropäolin² in die lipide Phase in Abhängigkeit von der Konzentration einiger charakteristischer Kationen in der wässrigen Schicht.

Unseres Erachtens kommt der Übergang von Tropäolin in die CCl₄-Phase dadurch zustande, dass sich vorerst das polybasische Protamin bzw. Polymyxin an der Grenzfläche Lipoid-Wasser anlagert. Dabei werden die sauren Gruppen des Phosphatids besetzt und an der Grenzfläche fixiert unter Freisetzung der Cholin-Komponente, an die sich der saure ionisierte Farbstoff anlagert und als Farbstoff-Lecithinkomplex in der lipiden Phase gelöst wird. Andererseits wird die Bildung des CCl₄-löslichen Trypaflavin-Lecithinsalzes durch die an der Grenzfläche adsorbierten polybasischen Verbindungen kompetitiv gehemmt.

Offensichtlich müssen 3 Bedingungen erfüllt sein, um maximale Wirkung zu erzielen: (1) Mehrfachbasische Verbindung, (2) sterisch wenig gehinderte stark basische Zentren (bei pH 7 zum grossen Teil protoniert) und (3) Adsorption an der Grenzfläche Lipoid-Wasser durch Van der Waalsche Kräfte.

Wir haben Verbindungen synthetisiert, die aller Voraussicht nach diese Bedingungen erfüllen sollten, und in unserer Versuchsanordnung gemessen. Eine Auswahl von Messresultaten zeigt Figur 3, wobei auch Verbindungen einbezogen sind, die die Bedingungen nicht erfüllen.

Es ist aus der Figur 3 ersichtlich, dass in der Tat maximale Hemmwirkung dann auftritt, wenn mindestens zwei stark basische Gruppen durch mehrere coplanare Ringe³ und durch Carbonamid-Gruppen verknüpft sind; ferner muss das Molekül eine durchgehende Konjugation aufweisen. Besonders interessant waren bisher Verbindungen mit folgender Struktur:



Diskussion. Es ist klar, dass solchen polyvalenten Basen auf Grund ihrer Fähigkeit, Phosphatide zu «fixieren», erhebliche Bedeutung als biologisch aktive Substanzen zukommt. Als «Phosphatid-Blocker» können sie als potentielle Hemmsubstanzen des Ionentransportes durch Phosphatidmembranen der lebenden Zelle angesehen werden. Da auch die Proteinsynthese über Phospho-Lipid-Aminosäure-Komplexe⁵⁻⁹ verläuft, könnte erwartet werden, dass solche polybasischen Stoffe durch Blockierung dieser Phospholipide einen Einfluss auf das Zellwachstum ausüben¹⁰. Nun sind in unserem Test die Lecithin-

moleküle innerhalb der lipoiden Phase und in der Grenzfläche frei beweglich. Ihre sauren Gruppen können sich innerhalb der Grenzfläche den basischen Zentren der adsorbierten Basen weitgehend anpassen.

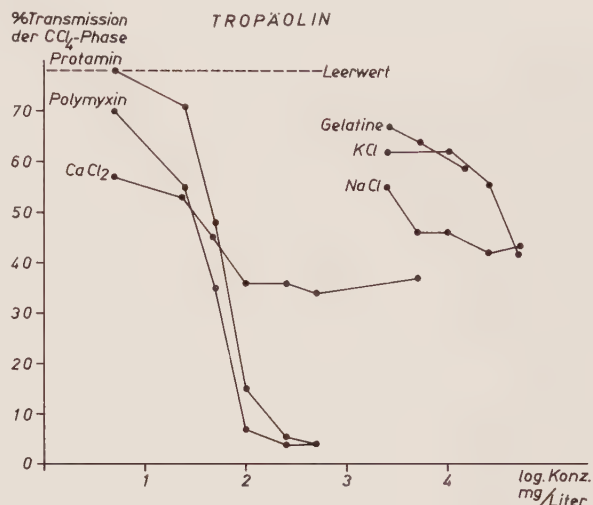


Fig. 2

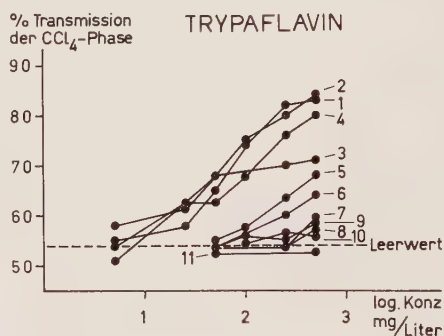


Fig. 3. (1) Terephthal-dianilid-4',4''-bis(2-imidazolin)-dihydrochlorid. (2) Terephthal-dianilid-3',3''-bis-(2-imidazolin)-dihydrochlorid. (3) Nitro-terephthal-dianilid-4',4''-bis(2-imidazolin)-dihydrochlorid. (4) Chlor-terephthal-dianilid-4',4''-bis(2-imidazolin)-dihydrochlorid. (5) Diphenyl-4,4'-bis(2-imidazolin)-dihydrochlorid. (6) Benzol-m-disulfonsäure-dianilid-4,4'-bis(2-imidazolin)-dihydrochlorid. (7) Äthylendiamin-dihydrochlorid. (8) Phenylen-1,4-bis(2-imidazolin)-dihydrochlorid. (9) Terephthal-bis-(N-methyl-piperazid)-dihydrochlorid. (10) Terephthal-dianilid-4',4''-bis-trimethylammoniumchlorid. (11) Putrescin-dihydrochlorid, Arcain-dihydrochlorid, Isophthal-bis-(γ-pyridylamid)-dihydrochlorid.

² Die relativen Konzentrationen der Farbstoffe in der CCl₄-Phase wurden wie früher (vgl. ¹) colorimetrisch bei 420 mμ bestimmt. Auch die übrige Messanordnung ist dieselbe.

³ Eine Parallele hierzu sind die Bedingungen, wie sie für das Vorkommen des kristallin-flüssigen Zustandes erfüllt sein müssen (vgl. ⁴).

⁴ CH. WIEGAND, in HOUBEN-WEYL, *Methoden der organischen Chemie*, 3. Aufl. (Stuttgart 1955), Bd. 3/1, p. 681. - D. VORLÄNDER, *Chemische Kristallographie der Flüssigkeiten* (Leipzig 1924). - C. WEYGAND, *Hand- und Jahrbuch der chemischen Physik*, 2 (Leipzig 1941). - CH. R. WIEGAND, *Z. Naturforsch.* 12b, 512 (1957).

⁵ J. A. V. BUTLER, G. N. GODSON und G. D. HUNTER, in R. J. C. HARRIES, *Protein Biosynthesis* (Academic Press, London 1961).

⁶ G. E. HUNTER und G. N. GODSON, *Nature* 189, 140 (1961).

⁷ R. W. HENDLER, *J. biol. Chem.* 234, 1466 (1959).

⁸ W. L. GABY und R. SILBERMAN, *Arch. Biochem. Biophys.* 87, 188 (1960).

⁹ G. N. GODSON und G. D. HUNTER, *Biochem. J.* 79, 37 P (1961).

¹⁰ Der Wirkungsmechanismus von natürlichen Antikörpern oder «endogenen Antibiotika»¹¹ könnte ebenfalls dieser Art sein.

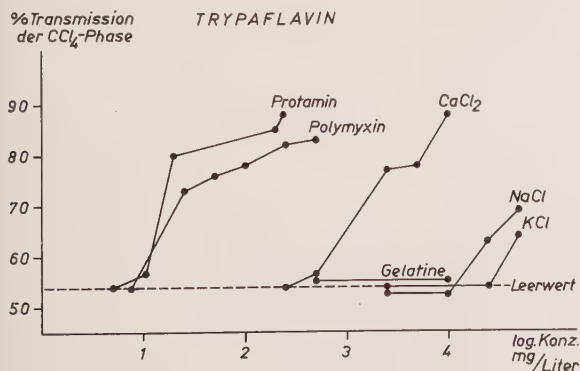


Fig. 1

Anders wird jedoch die Situation, wenn man wie STEINBERG¹² annimmt, dass die sauren Phosphatidgruppen an der Grenzfläche der Zellen in einer starren, zellspezifischen mosaikartigen Struktur verteilt sind. Dann wird zusätzlich auch noch die Lage der positiven Ladungen dieser Hemmsubstanzen eine wesentliche Rolle spielen, und zwar in dem Sinne, dass zu einer bestimmten Anordnung der Phosphatide in der Zellwand die Lage der basischen Gruppen in der Molekülebene kongruent sein muss. Nur so wird durch potenzierte Coulombsche Attraktion eine maximale Affinität gewährleistet. Unser Test zeigt nur die allgemeinen Bedingungen einer Phosphatid-Blockierung. Eine zellspezifische Wirkung, die durch geeignete Variation der Strukturen erwartet werden kann, muss am biologischen Objekt eruiert werden.

Es hat sich nun tatsächlich gezeigt, dass eine Reihe von neuen Verbindungen, die nach diesen Gesichtspunkten synthetisiert wurden, in Übereinstimmung mit der Wirkung an unserem Test eine auffällige und differenzierte Wirkung auf lebende Zellen haben. So übertreffen einige dieser Stoffe die *in vitro*-Hemmwirkung von INH auf Tbc-Bakterien. Besonders interessant ist jedoch die Wirkung bei tierexperimentellen Neoplasmen. So verlängert die oben mit der Strukturformel angeführte Verbindung die Überlebenszeit von mit Leukämie beimpften Mäusen bei grosser therapeutischer Breite um 330% bei $R = H$, um 250% bei $R = NO_2$ und um 400% bei $R = Cl$ ¹³. Die Wir-

kung ist spezifisch auf jeweils eine Krebsart. Durch kleine Variationen in der Struktur wird die Wirkung auf andere Neoplasmen, wie Sarkom oder Carcinom, verschoben.

Wir sind damit beschäftigt, eine grössere Zahl von strukturell verwandten Verbindungen herzustellen und zu prüfen¹⁴.

Summary. Based on the behaviour of lecithin in a bio-physical cell model, novel compounds have been developed which are regarded as 'phosphatide blockers' in the membrane of the living cell. They inhibit neoplasms in animal experiments to a high degree, and are therefore potential chemotherapeutic agents of great interest.

R. HIRT und R. BERCHTOLD

Forschungsinstitut Dr. A. Wander AG, Bern (Schweiz),
21. Juli 1961.

¹¹ H. FISCHER und K. RÖWE, Trans. 6th Congr. Europ. Soc. Haematology (Copenhagen 1957), p. 927.

¹² M. S. STEINBERG, Amer. Naturalist 92, 65 (1958).

¹³ Wir verdanken diese Angaben dem Cancer Chemotherapy National Service Center (USA).

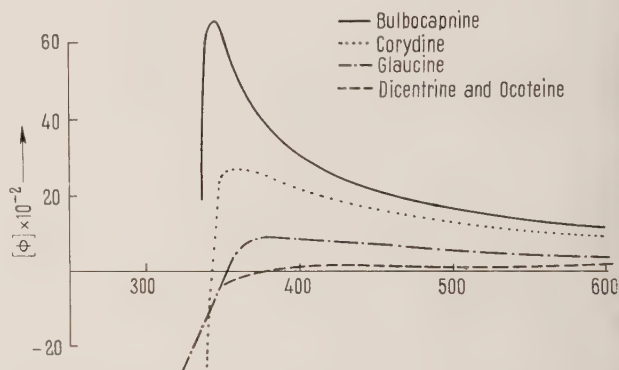
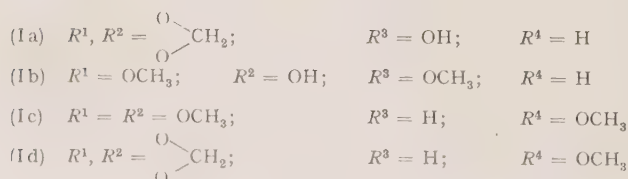
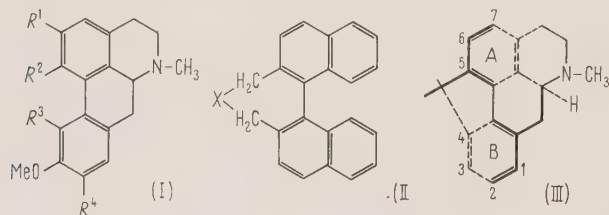
¹⁴ Wir danken Herrn H. HÄNNI für die exakte und zuverlässige Ausführung eines Teiles der experimentellen Arbeiten.

The Anomalous Rotatory Dispersion Curves of Aporphine Alkaloids

The configuration of optically active biaryls has been successfully correlated by examining their rotatory dispersion curves^{1,2}. We wish now to report some results of our studies on the rotatory dispersion of some aporphine alkaloids which may be connected with their biphenyl systems.

Bulbocapnine (Ia) and corydine (Ib) exhibit anomalous positive curves (Figure) which are somewhat similar to those presented by members of the (S)-series of 2,2'-bridged, 1,1'-binaphthyls¹ (II). Bases which have the 4 position unsubstituted, such as glaucine (Ic), dicentrine (Id), and possibly ocoteine³, nevertheless show plain curves (or nearly so as in the case of glaucine, where there is a plateau at about 365–370 mμ).

Recently, SHAMMA⁴ has called attention to the striking correlations⁵ between the optical rotations (D line) and the ultraviolet spectra of the aporphine alkaloids. The absolute stereochemistry of the asymmetric carbon of the latter has been shown to be as indicated in (III)⁶, where the rigid biphenyl systems is fixed in a (S) configuration and the substituents in the ring A are β (above the plane)⁴.



It would appear that the anomalous curves obtained for bulbocapnine and corydine are associated with strained bi-phenyl systems, and that the differences of magnitude of the optical rotations (D line)⁴ and of the rotatory dispersion curves are related to the change of their dihedral angles between the planes of the two aromatic rings caused by the presence or absence of substituents at carbon 4⁷.

Zusammenfassung. Die Untersuchung der Rotationsdispersionskurven einiger Aporphin-Basen ergab anomale Dispersion für Basen, die in 4-Stellung substituiert sind, und normale Dispersion für die unsubstituierten Basen.

M. J. VERNENGO⁸

University Chemical Laboratory, Cambridge (England),
April 17, 1961.

¹ K. MISLOW, M. A. W. GLASS, R. E. O'BRIEN, P. RUTKIN, D. H. STEINBERG, and C. DJERASSI, J. Amer. chem. Soc. 82, 4740 (1960).

² K. MISLOW and C. DJERASSI, J. Amer. chem. Soc. 82, 5247 (1960).

³ Unpublished results.

⁴ M. SHAMMA, Exp. 16, 484 (1960).

⁵ A. GIRARDET, J. chem. Soc. 1931, 2630.

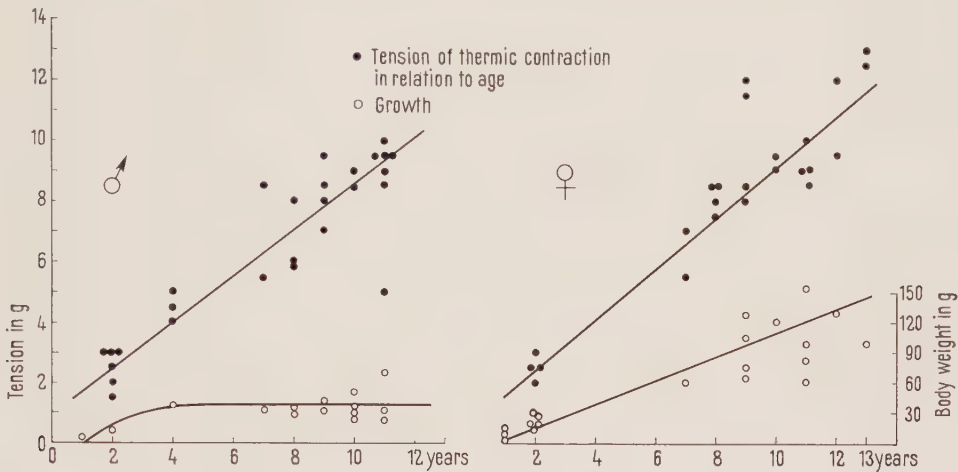
⁶ H. CORRODI and E. HARDEGGER, Helv. chim. Acta 39, 889 (1956).

⁷ The author is indebted to Prof. W. KLYNE and Dr. J. HARLEY-MASON for their comments.

⁸ Present address: Laboratorio de Química Orgánica, Facultad de Ciencias Exactas y Naturales, Buenos Aires (Argentina).

The Ageing of *Xenopus laevis*

Xenopus laevis, the South African claw frog, is often used for laboratory tests of gonadotrophic hormones, etc. Males are fully grown at 2 years of age and then maintain a weight of about 60 g. Females, on the contrary, continue to grow and, at the age of 12–13 years, they have a body weight of 100–150 g (Figure).



We tested whether collagen aged in these frogs in a similar way as in mammals. It had been shown¹ in the latter that, during thermic contraction (shrinkage), the isometric tension of tendon fibres, consisting of practically pure collagen, increases with age. This is explained as a consequence of the increase of cross-linkages in the collagen macromolecules with age. The fact that the quantity of hydroxyprolin-containing complexes which are liberated during thermic contraction, decreases with age, is a confirmation of the increased cross-linking in aged collagen².

These facts were originally discovered on the tail tendons of rats, and for hydroxyprolin also on cow-hide and human skin³.

We have now shown that in *Xenopus laevis* the same changes appear in the collagen of the tendons as in mammals. The tension of thermic contraction increases from 2.5 g in animals of 2 years of age to 10–11 g in 12–13 year old ones. These characteristic age changes are independent of the growth and the weight, which in old individuals are different for males and females, as seen in the Figure.

Thus the tension of the thermic contraction of tendon-(collagen)-fibres is a real measure of 'biological age' also in *Xenopus laevis*.

A detailed description will be given in 'Gerontologia'⁴.

Zusammenfassung. (1) Die Spannung, welche sich bei der thermischen Kontraktion einer Sehne entwickelt, ist charakteristisch für das Alter der Tiere. Daraus lassen sich

mit dem Alter fortschreitende Veränderungen des Collagens erkennen. (2) Bei *Xenopus laevis* zwischen 2 und 13 Jahren nimmt mit zunehmendem Alter dieser Wert, ebenso wie bei Säugetieren, zu, unbeeinflusst davon, dass das Wachstum der Weibchen viel grösser als das der Männchen ist.

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¹ F. VERZÁR, *Gerontologia* 1, 363 (1957); 2, 81 (1958).
² A. MEYER and F. VERZÁR, *Gerontologia* 3, 288 (1959).
³ F. VERZÁR, *Gerontologia* 4, 105 (1960).
⁴ We thank Prof. F. E. LEHMANN in Bern and Prof. A. C. J. BURGERS in Utrecht for many animals.
This work was supported by the Muscular Dystrophy Association of America.

The Age Parameter of Pharmacological Activity

Toxic or pharmacological activity of drugs is generally tested on young animals only. We have recently made parallel measurements on rats of different ages from the same inbred colony. A number of different pharmacies were used and ten animals were always compared for each concentration and age group.

No age differences were found with substances with peripheral nerve ending activity, such as *Acetylcholin* and *Histamin*. They were tested on the surviving intestine of guinea pigs (Magnus preparation) with animals of the age of 2, 20, and 50–70 months.

Similarly the antagonistic inhibition of these with *Atropin* or *Sandosten* were unchanged in old age, as is shown in the Table.

Age months	Number of animals with threshold activity			Number of animals with inhibitory effect	
				Atropin	
	Acetylcholin			2.5 · 10 ⁻⁸	1.125 · 10 ⁻⁸
	64 · 10 ⁻⁸	128 · 10 ⁻⁸			
2	6	4		2	8
20	5	5		1	9
50–70	4	6		5	5
	Histamin			Sandosten	
	1.28 · 10 ⁻⁹	2.56 · 10 ⁻⁹	5.12 · 10 ⁻⁹	25 · 10 ⁻⁶	1.25 · 10 ⁻⁶
2	1	7	3	5	5
20	4	4	2	4	6
50–70	3	4	3	4	6

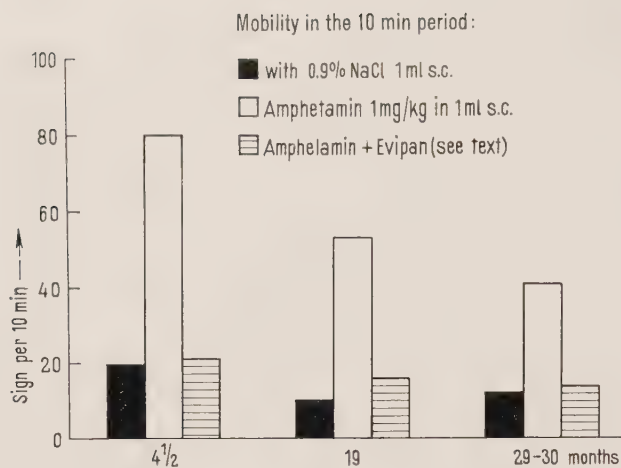


Fig. 1

Substances which act upon the central nervous system behave in a very different way. The narcotic action of *Hexobarbital* (Evipan) with i.p. injections was complete with 80 mg/kg in 4 to 5 months (i.e. young) rats, whereas in 29–32 months old rats even 50 mg/kg gave complete narcosis.

The central excitant *Amphetamine* (Benzedrine) in a dose of 1 mg/kg produces spontaneous motor excitation of a high degree and long duration in young, and much less and shorter in aged rats, as Figure 1 shows.

Also the antagonistic inhibition of the motor excitation by *Hexobarbital* is attained with 90 mg/kg in 4–5 months old animals, while in old animals of 19 months 40 mg/kg, and of 29–31 months only 30 mg/kg was needed.

The same differences were found for the psychomotor stimulant *Ritalin* (10 mg/kg s.c.) and the inhibitory effect on it with *Regitin*. The latter inhibits in doses of 50 mg/kg in 4–5 months old rats, and 30 mg/kg in 29–31 months old animals.

Thalamic centres of old animals also show a decreased sensitivity with ageing. The dosage of *Procaïn* (Novocain) (i.m. 10 mg/100 g) which decreases the body temperature by 1.5°C in young guinea pigs, decreases it by 2°C in 3 1/2 and 6 1/2 years old animals. More characteristic is that normal body temperature was reached again after 110 min in 6 months old and after 210 min in the 3 1/2 and 6 1/2 years old animals.

Similarly, the centre which regulates food intake by appetite and hunger, is much more influenced by a dose of 1 mg/kg *Preludin* in 29–31 months old rats (causing 35% decrease of food intake) than in 11 months old animals,

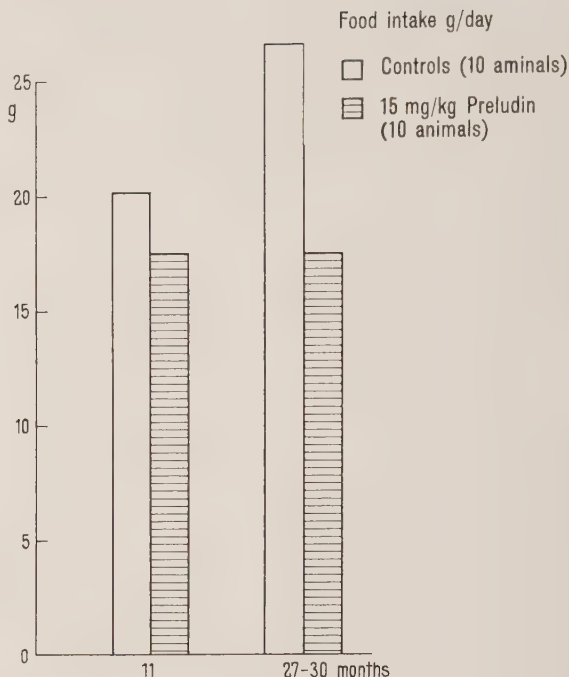


Fig. 2

where the food intake was decreased only 12%. Similar results were obtained with *Benzedrine* (Figure 2).

For an explanation of these changes with age, it might be remembered that for different parts of the central nervous system a decrease of the quantity of grey matter, i.e. of nerve cells, has been shown in old animals. This may be the cause of the diminished activity of stimulants and an increase of inhibitory influences of substances with depressive action.

A detailed description is given in 'Gerontologia'.

Zusammenfassung. Je nach dem Alter wirken Pharmaka sehr verschieden. Hexobarbital ist bei alten Tieren stärker narkotisch; Amphetamin und Ritalin weniger erregend; Amphetamin und Preludin wirken stärker appetithemmend und nach Novokain ist die Hemmung der Wärme-regulation stärker bei alten Tieren. Peripher angreifende Pharmaka zeigen keine Unterschiede.

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Action d'une irradiation générale sur les échanges d'eau entre le sang et la cavité gastrique pendant l'absorption du glucose

Dans un travail antérieur¹ nous avons constaté qu'une irradiation générale par les rayons X ralentit l'absorption intestinale du glucose. Ayant calculé que ce ralentissement n'est dû qu'en partie à la diminution de tolérance aux glucides qui se développe au même moment^{2,3}, nous avons exploré un métabolisme très radiosensible⁴ qui joue un rôle important dans l'absorption intestinale^{5,6}, le métabolisme de l'eau, en nous limitant dans cette note à ce qui se passe dans l'estomac après l'ingestion d'une dose

unique de glucose (1100 mg dans une solution à 135 mg/cm³). Nos expériences ont porté sur 30 cobayes

¹ M. LOURAU et O. LARTIGUE, Arch. Sci. Physiol. 5, 83 (1951).

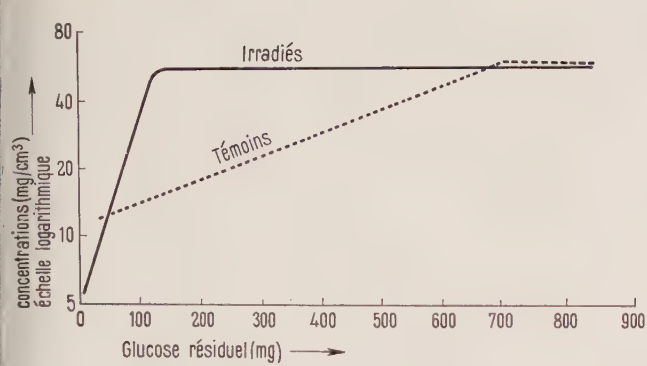
² M. LOURAU et O. LARTIGUE, Arch. Sci. Physiol. 4, 197 (1950); Exper. 7, 428 (1951).

³ M. LOURAU, Arch. Sci. Physiol. 13, 29 (1959), et résultats inédits.

⁴ G. VAN DEN SCHUEREN et J. BONTE, Actes de la deuxième Conférence internationale sur l'utilisation de l'énergie atomique à des fins pacifiques (Genève 1958). Nations Unies, Genève, 10, 558 (1959).

⁵ M. B. VISSCHER, E. S. FECHTER, C. W. CARR, H. P. GREGOR, M. BUSHEY et D. E. BARKER, Amer. J. Physiol. 142, 550 (1944).

⁶ H. H. USSING, Adv. Enzymol. 13, 21 (1952).



témoins et 32 cobayes irradiés totalement depuis 14 jours par une dose sublétales de rayons X (500 roentgens de 250 kV) et nous avons utilisé pour mesurer le volume de la solution gastrique et sa teneur en glucose les techniques décrites en ³.

Chez les témoins, le glucose ingéré, qui ne passe dans l'intestin que par petites fractions, reste dans l'estomac où il est dilué par les sécrétions de la muqueuse. Cette dilution se fait en deux temps: un premier temps, très court (moins de 15 min) abaisse la concentration à 60 ± 12 mg/cm³, puis il y a un arrêt; le glucose franchit le pylore sans que la concentration change jusqu'à ce qu'il ne reste plus que 700 mg de glucose dans l'estomac. A ce moment, la concentration baisse de nouveau en suivant une loi définie: entre le log des concentrations et le glucose résiduel il y a une corrélation hautement significative (coefficient de corrélation 0,91; $p = 0,001$; voir Figure et première partie du Tableau).

L'irradiation modifie la marche des phénomènes. La première dilution reste normale, aussi rapide, aussi importante. La concentration atteinte: 56 ± 10 mg/cm³ ne diffère pas statistiquement de celle des témoins (t de Fisher = 1,9), mais elle persiste jusqu'à ce que l'estomac ait évacué à peu près tout son contenu; c'est seulement lorsqu'il ne reste plus que 100 mg de glucose que la concentration s'abaisse de nouveau, mais elle le fait en suivant la loi normale. Le coefficient de corrélation 0,77 est significatif au seuil de probabilité 0,01, mais la droite représentant la fonction: log concentrations/glucose résiduel a une pente 8 fois plus forte que chez les témoins (Figure et deuxième partie du Tableau).

Pendant une longue période, les conditions d'absorption sont donc fortement perturbées chez les animaux irradiés, car c'est essentiellement la dilution que la

	Témoins	Irradiés
Glucose résiduel (mg)	1100 à 700	1100 à 100
Concentrations (mg/cm ³)	60 ± 12	56 ± 10
Effectifs	14	16
$(t = 1,9)$		
Glucose résiduel (mg)	700 à 0	100 à 0
Coefficient de corrélation	0,91	0,77
Probabilité	0,001	0,01
Pente de la droite de régression y en x	$1 \cdot 10^{-3}$	$8 \cdot 10^{-3}$
Effectifs	16	16

solution ingérée subit dans l'estomac qui règle la vitesse de l'absorption intestinale^{3,7}. Toutefois il n'est pas ici possible d'estimer l'importance, voire même le sens de cette perturbation dont les conséquences sont multiples, mais opposées. Alors que la persistance d'une concentration élevée accélère l'absorption⁷, d'autres facteurs agissent en sens inverse; citons, l'absence d'eau, peut être aussi une moindre perméabilité à l'eau de la muqueuse intestinale analogue à celle que l'on a observée pour le péritoine⁸, la diminution du volume gastrique, qui ralentit le transit pylorique⁹.

L'intérêt des observations ci-dessus est de rattacher à un problème très général: perturbation radioinduite du métabolisme hydrique, un fait qui paraissait tout à fait étranger: la diminution de l'absorption intestinale des glucides.

Les droites de régression représentées dans la Figure ont été tracées par la méthode des moindres carrés. Le Tableau contient les statistiques relatives aux données expérimentales.

Summary. A sublethal dose of X-rays, delivered to guinea-pigs 14 days before a glucose absorption test, alters the pattern of water exchange between the blood and the gastric cavity. The normal dilution, which proves to be a main factor in the regulation of the intestinal absorption of sugar, is no longer achieved.

M. LOURAU

Institut de Biologie, Paris (France), le 20 décembre 1960.

⁷ M. LOURAU, *Exper.* 15, 192 (1959).

⁸ P. MEYNIÉ, Thèse de Doctorat en médecine (Bordeaux 1950), (Drouillard Ed.).

⁹ J. N. HUNT et I. MACDONALD, *J. Physiol.* 126, 459 (1954).

PRO LABORATORIO

Utilization of the pH-Stat in the Study of Cellular Metabolism

The buffering properties of the systems employed in the study of cellular metabolism prevent appreciable changes of hydrogen ion concentration in the medium; therefore, in their presence, we cannot recognize directly the release of acid radicals and in brief the appearance of the protons produced by the cells as terminal step of their metabolic process.

In this note we report preliminary results on the study of cellular metabolism carried out on the basis of changes of hydrogen ion concentration in unbuffered solutions, as measured by an automatic titrator adapted as pH-stat¹.

We made use of a Titrigraph mod. TTT 1 A (Radiometer Copenhagen N.V. Denmark) with some modifications. The titration chamber was immersed in a thermostatically controlled bath; it was closed by a loose rubber stopper through which passed the two pH electrodes, the alkali delivery tip, the gas delivery tip and the glass rod of an electric stirrer. A low stirring speed of about 150 r.p.m. was chosen, to avoid damaging the cells. A magnetic stirrer could alternatively have been used. A hole in the stopper made possible the withdrawal of samples during the experiments.

These preliminary experiments were carried out with Yoshida's ascites cells and rat liver slices.

¹ C. F. JACOBSEN and J. LÉONIS, *C. R. Trav. lab. Carlsberg, Sér. chim.* 27, 333 (1951).

The ascitic fluid was suspended in 5 vol of isotonic saline solution and centrifuged twice at 4°C for 10 min at $350 \times g$, discarding the supernatant. The cells were then resuspended in the following medium: NaCl 0.154 Mol/100, KCl 0.154 Mol/4, CaCl_2 0.11 Mol/3, MgSO_4 0.154 Mol/1, KH_2PO_4 0.20 Mol/0.5, NaOH 0.1 N added until pH 7.4.

After centrifuging twice, the cells were resuspended in the same medium in the proportion of 1 vol packed cells to 5 vol of medium. The packed cell volumes were determined in a hematocrit after centrifuging for 20 min at $2500 \times g$. In some instances, dry weight determinations were also made. The liver slices were withdrawn and prepared in the usual way². The titration vessel usually contained 12 ml of medium and 3 ml of cellular suspension (total number of cells about 150×10^6) or 15 ml of medium and about 60 mg of liver slices (dry weight). The solution was adjusted to the desired value of pH 7.4 with small quantities of NaOH N/50, and the experiments started as soon as temperature equilibrium was reached (2–3 min). The temperature of the bath was maintained at 37°. The concentration of the alkali used in the titration was of 0.02 normal, sufficiently low to avoid appreciable cell damages.

Before introduction of the cells, the liquid medium was saturated with gas and during the course a stream of a gas was blown over the surface of the liquid. Bubbling was avoided to prevent foaming.

Since the lysis of cells releases materials with buffering properties, we checked frequently the amount of lysed cells at the end of the experiments. The optical densities at 260 and 280 m μ showed that the percentage of cells lysed was close to that observed in the Warburg vessels (about 1.5%). The rate of cellular respiration, in absence of any substrate, is given by the amount of alkali required to neutralize the protons derived from the first dissociation of carbonate according to the reaction: $\text{CO}_2 + \text{H}_2\text{O} \rightarrow \text{H}_2\text{CO}_3 \rightarrow \text{H}^+ + \text{HCO}_3^-$ and this is recorded by the pH-stat.

The Figure 1 shows a typical titration curve obtained with Yoshida's ascites cells in the presence of oxygen. When ascites cells are tested in presence of a substrate (glucose) under aerobic conditions, there are two sources of protons, carbonate and lactic acid. In absence of oxygen, there is only one source, namely lactic acid (Figure 2).

In the case of aerobic glycolysis, we made a correction of the amount of alkali required to neutralize the protons derived from carbonate, on the basis of the Crabtree effect (Figure 1 – dashed line). Since this correction was found to be very constant in different experiments, this value needs to be determined by the manometric measurements only once for the system under investigation.

The quantities of lactic acid released during the glycolysis, as estimated by colorimetric³ and enzymatic⁴

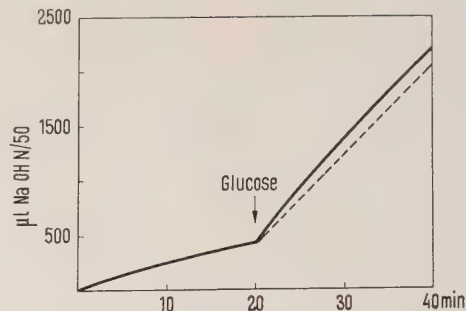


Fig. 1. Titration curve for Yoshida's ascites cells. Experiment No. 12. Gas: oxygen. $T = 37^\circ\text{C}$. Cells 97.5 mg dry weight, glucose μMol 210. (The dashed line represents the correction of the glycolysis on the basis of the Crabtree effect (57%).)

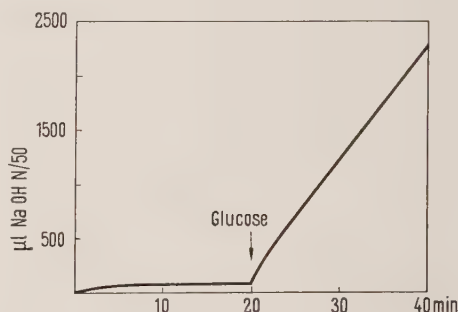


Fig. 2. Titration curve for Yoshida's ascites cells. Experiment No. 12. Gas: argon. $T = 37^\circ\text{C}$. Cells 97.5 mg dry weight, glucose μMol 210.

methods, are very close to those calculated from the equivalents of alkali delivered.

The Table shows data on the respiration and glycolysis of Yoshida's ascites cells and rat liver slices, calculated both from titrimetric and manometric measurements. The values reported above can also be expressed in equivalents of proton neutralized. On this basis, the CO_2 released by Yoshida's ascites cells corresponds to 0.254×10^{-6} g protons/mg/h, the aerobic glycolysis to 1.00×10^{-6} and the anaerobic glycolysis to 1.4×10^{-6} g protons/mg/h from lactic acid.

On the basis of these experiments, it seems that the use of a pH-stat can improve the study of cellular metabolism by making it possible to modify such factors as temperature or the nature of the gas, to introduce different substrates, and to withdraw samples during the course of the experiments. It also greatly increases the speed of the measurements since only a few minutes are required to obtain adequate and reproducible results. The experiments can also be performed with large quantities of material and, since the high sensitivity of the instrument permits the detection of very small quantities of protons, it becomes possible to investigate the first stages of metabolic activity after the addition of a substrate.

	Titrimetric method			Manometric method		
	Q_{CO_2} ^a	μMol $L(+)$ lactic acid ^b		Q_{CO_2} ^a	μMol $L(+)$ lactic acid ^b	
		I ^c	II ^d		I ^c	II ^d
Yoshida's cells	5.7	1.0	1.4	6.6	1.2	1.4
Rat liver slices	4.06	—	—	4.25	—	—

^a The amount of CO_2 is given in mm³ at standard pressure and temperature per mg dry weight of tissue per h.

^b This value means the μMol of $L(+)$ lactic acid produced per mg dry weight of tissue per h.

^c In the presence of oxygen.

^d In the presence of argon.

² W. W. UMBREIT, R. H. BURRIS, and J. F. STAUFFER, *Manometric Techniques* (Burgess Publishing Co. 1957).

³ S. B. BARKER and W. H. SUMMERSON, *J. biol. Chem.* **138**, 535 (1941).

⁴ G. PFLEIDERER and K. DOSE, *Biochem. Z.* **326**, 437 (1955).

Riassunto. È stata studiata la possibilità di seguire con un titolatore automatico impiegato come pH-stat l'andamento della respirazione e della glicolisi di cellule sopravvivenenti. I valori ottenuti sono in accordo con quelli descritti nella letteratura.

Rispetto al metodo manometrico sono segnalati alcuni vantaggi quali la rapidità delle determinazioni, la possi-

bilità di variare più parametri sperimentali nel corso dell'esperienza nonché la facilità di prelievo di materiale durante l'esperimento.

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Istituto Regina Elena per lo Studio e la Cura dei Tumori, Roma (Italy), March 6, 1961.

STUDIORUM PROGRESSUS

An Approach to Molecular Dynamics of Chemical Reaction

According to the theory of transition state¹, the reaction rate constant can be expressed as

$$k = \kappa (kT/h) \{Q_{(t)}/Q_{(i)}\} \exp(-\Delta U_i/kT), \quad (1)$$

where κ is the transmission coefficient, kT the statistical temperature which is the absolute temperature T multiplied by Boltzmann's constant k , h Planck's constant, $Q_{(t)}$ the partition function of the so-called transition state, $Q_{(i)}$ the partition function of the initial molecular system, and ΔU_i is the activation energy. The chemical reaction has been classified by HORIUTI into the two types, the one being an effusion-type and the other a diffusion-type, by the relations of the transmission coefficient $\kappa \simeq 1$ and $\kappa \ll 1$, respectively².

However, κ is a correction factor brought somewhat formally into the expression of rate constant. For the reaction of effusion-type ($\kappa \simeq 1$), it can be considered that the transition state method of Eyring's is a good approach. But it must be supposed that the Eyring's theory is inapplicable to the reaction of diffusion-type, because the method is based on the equilibrium model of chemical reaction whose original concept can be found in the classical treatment of ARRHENIUS³. Therefore, it will be desirable to deal with the problem from the more general point of view.

This paper describes an approach to the non-equilibrium model of chemical reaction from the molecular dynamical theory. One of our purposes in this work is to find the mutual relationships among the several theoretical treatments of reaction rate from a unified and general situation.

In the approach based on the non-equilibrium model, the problem is reduced to the diffusion problem of particles over a potential barrier from a potential-hollow corresponding to the initial configurations of reaction system to another hollow belonging to the final configurations of product system on the adiabatic potential energy surface⁴. If the degree of freedom of the reacting molecular system is given by f , the transition (hyper-) surface of the dimension $(f-1)$ can be defined in the vicinity of the 'watershed' of a potential barrier. A profile of the potential energy surface in the two dimensional case is shown diagrammatically in Figure 1. Our interest is focused upon the diffusion flux of the representative points of reaction system across the transition surface in phase space as a consequence of Brownian motion. It may be safely said that Brownian motion in the molecular level is responsible for diffusion process in the macroscopic level, in a broad sense. As shown in Figure 2, the behaviour of the flow of the representative points can be likened to the motion of Brownian particles as a whole. Such a Brownian particle-like motion results from the irregularly fluctuating forces due to the interaction between the reaction system and the surroundings. The reacting system is presumed to accept or lose energy through the fluctuat-

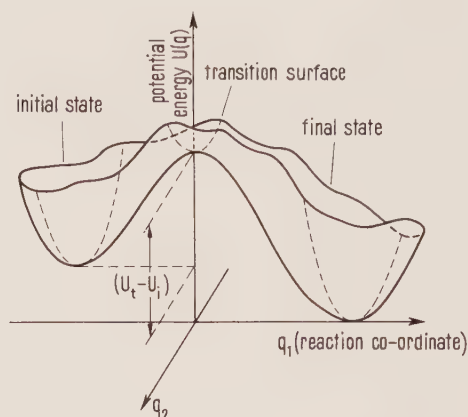


Fig. 1. A profile of two-dimensional potential energy surface.

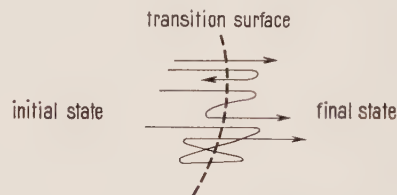


Fig. 2. Brownian particle-like motion of the representative points in the neighbourhood of the transition surface.

ing forces from the surroundings. Then the passage of the reacting system in the vicinity of the top of potential barrier to the final state taking the molecular configurations of product can be achieved by means of a large number of fluctuations.

In the f -dimensional phase space, the co-ordinates of position are called $(q_1, q_2, \dots, q_f)$, the conjugate momenta are $(p_1, p_2, \dots, p_f)$, and the reduced masses with regard to those degrees of freedom are $(M_1, M_2, \dots, M_f)$, respectively. The co-ordinates are chosen so that these normalize each other in the neighbourhood of the transition surface, and q_1 is selected as the reaction co-ordinate.

The Hamiltonian H_r of the reaction system is given by

$$H_r = \sum_{\alpha=1}^f p_{\alpha}^2 / 2 M_{\alpha} + U(q_1, q_2, \dots, q_f), \quad (2)$$

where $U(q_1, q_2, \dots, q_f)$ is the potential energy of the system.

The potential energy of the system is developed in the power series of the displacements around the saddle-point in the transition surface, whose molecular configuration is specified by suffix t , as the following:

¹ S. GLASSTONE, K. J. LAIDLER, and H. EYRING, *Theory of Rate Processes* (McGraw-Hill, New York 1941).

² J. HORIUTI, *Pap. Inst. Phys. Chem. Res. Tokyo* 34, 1174 (1938); *Bull. Chem. Soc. Japan* 13, 210 (1939).

³ S. ARRHENIUS, *Z. phys. Chem.* 4, 226 (1889).

⁴ H. A. KRAMERS, *Physica* 7, 284 (1940).

$$U(q_1, q_2, \dots, q_f) = U(q_{1t}, q_{2t}, \dots, q_{ft}) + \sum_{\alpha, \beta=1}^f C_{\alpha\beta} \Delta q_\alpha \Delta q_\beta + \dots \quad (3a)$$

where $\Delta q_\alpha \equiv (q_\alpha - q_{\alpha t})$. Since the position co-ordinates are normal in the vicinity of the transition surface, the $C_{\alpha\beta}$ matrix has only the diagonal element:

$$U(q_1, q_2, \dots, q_f) = U(q_{1t}, q_{2t}, \dots, q_{ft}) + \sum_{\alpha=1}^f C_{\alpha\alpha} (\Delta q_\alpha)^2 + \dots \quad (3b)$$

All the elements but the C_{11} are positive, because of q_1 being the reaction co-ordinate. The C_{11} element can be put as

$$C_{11} \equiv - (1/2) (\partial^2 U / \partial q_1^2)_{\Delta q_1=0} = - (1/2) \omega_t^2 M_1, \quad (3c)$$

where ω_t has the dimension of frequency (multiplied by 2π). All the other terms are collectively called

$$\tilde{U}(q_2, q_3, \dots, q_f) = \sum_{\alpha=2}^f C_{\alpha\alpha} (\Delta q_\alpha)^2. \quad (3d)$$

After all, Eq. (3b) becomes

$$U(q_1, q_2, \dots, q_f) = U_t - (1/2) \omega_t^2 M_1 x^2 + \tilde{U}(q_2, q_3, \dots, q_f) + \dots, \quad (4)$$

where $U_t \equiv U(q_{1t}, q_{2t}, \dots, q_{ft})$, and $x \equiv \Delta q_1 = (q_1 - q_{1t})$.

The KRAMERS-CHANDRASEKHAR'S^{4,5} equation governing the time variation of $W(q_1, q_2, \dots, q_f, p_1, p_2, \dots, p_f; t)$, which is the distribution function for the molecular system of f -degrees of freedom, is written as

$$\{\partial/\partial t + F_\alpha \partial/\partial p_\alpha + \langle p_\alpha/M_\alpha \rangle \partial/\partial q_\alpha\} W = \partial/\partial p_\alpha \{kT \zeta_{\alpha\beta} \partial W/\partial p_\beta + \zeta_{\alpha\beta} \langle p_\beta/M_\beta \rangle W\}, \quad (5)$$

where $F_\alpha = -\partial U/\partial q_\alpha$, and $\zeta_{\alpha\beta}$ is the friction tensor:

$$\zeta_{\alpha\beta} = (1/kT) \int_0^\infty \overline{A_\alpha(t) A_\beta(t+s)} ds, \quad (6)$$

A_α is the component of the total fluctuating force owing to the interaction between the α -th degree of freedom of the reaction system and the surroundings, and the bar means an ensemble average with respect to the initial co-ordinates and momenta of all the other molecular systems.

Provided that all the $\zeta_{\alpha\beta}$ except the ζ_{11} vanish, the component along the reaction co-ordinate of the friction tensor, and under the condition of Eq. (4), W becomes separable as the following:

$$W(q_1, q_2, \dots, q_f, p_1, p_2, \dots, p_f; t) = W_1(q_1, p_1; t) \cdot W'(q_2, q_3, \dots, q_f, p_2, p_3, \dots, p_f; t), \quad (7)$$

where $W_1(q_1, p_1; t)$ is the solution of the equation

$$(\partial/\partial t + F_1 \partial/\partial p_1 + p_1/M_1 \partial/\partial q_1) W_1 = \partial/\partial p_1 (kT \zeta_{11} \partial W_1/\partial p_1 + \zeta_{11} p_1/M_1 W_1). \quad (8)$$

In the neighbourhood of the transition surface, Eq. (8) is reduced to

$$(\partial/\partial t + \omega_t^2 M_1 x \partial/\partial p_1 + p_1/M_1 \partial/\partial x) W_1 = \partial/\partial p_1 (kT \zeta_{11} \partial W_1/\partial p_1 + \zeta_{11} p_1/M_1 W_1). \quad (9)$$

Here we have used Eq. (4) for F_1 .

Admitting that the difference of the potential energies between the top of barrier and the bottom of the hollow of the initial state, $(U_t - U_i)$, is sufficiently larger than the thermal energy kT , we can consider the steady diffusion flux in the neighbourhood of the transition surface.

Eq. (9) becomes thereby

$$(\omega_t^2 M_1^2 x \partial/\partial p_1 + p_1 \partial/\partial x) W_1 = \partial/\partial p_1 (kT \zeta_{11} M_1 \partial W_1/\partial p_1 + \zeta_{11} p_1 W_1). \quad (10)$$

According to the treatment of CHANDRASEKHAR'S⁵, the solution of Eq. (10) can be obtained in the form of

$$W_1 = N_1 \cdot f(s) \cdot \exp[-\{p_1^2/2 M_1 - (1/2) \omega_t^2 M_1 x^2 + U_t\}/kT], \quad (11a)$$

$$f(s) = \{(a - \zeta_{11})/2\pi kT \zeta_{11} M_1\}^{1/2} \times \int_{-\infty}^s \exp\{- (a - \zeta_{11}) s^2/2 kT \zeta_{11} M_1\} ds, \quad (11b)$$

$$\text{and } a = (\zeta_{11}/2) + \{(\zeta_{11}/2)^2 + (\omega_t M_1)^2\}^{1/2}, \quad (11c)$$

where N_1 is a normalization constant, and $s \equiv (p_1 - a x)$.

The boundary conditions are set up for Eq. (11a) as the following:

$$\left. \begin{aligned} f(s) &\rightarrow 1 & \text{as } s &\rightarrow +\infty \\ f(s) &\rightarrow 0 & \text{as } s &\rightarrow -\infty \end{aligned} \right\}, \quad (12)$$

these result from the initial condition that the system under consideration exists in the equilibrium initial state at first and not in the final state.

The steady diffusion flux $J_{st}^{(1)}$ across the transition surface, whose position is given by $x = (q_1 - q_{1t}) = 0$, can be calculated from

$$J_{st}^{(1)} = \int_{-\infty}^{+\infty} W_1(x=0, p_1) p_1/M_1 dp_1. \quad (13)$$

By making use of Eqs. (11a, b), we obtain finally

$$J_{st}^{(1)} = N_1 \{(a - \zeta_{11})/2\pi kT \zeta_{11} M_1\}^{1/2} \exp(-U_t/kT) \times \int_{-\infty}^{+\infty} dp_1 p_1/M_1 \exp(-p_1^2/2 kT M_1) \int_{-\infty}^{p_1} ds \exp\{- (a - \zeta_{11}) s^2/2 kT \zeta_{11} M_1\} = kT N_1 \{1 - (\zeta_{11}/a)\}^{1/2} \exp(-U_t/kT). \quad (14)$$

We shall proceed to make partial use of the concept of Eyring's, that is the equilibrium assumption with regard to the $(f-1)$ degrees of freedom except a single one of the reaction co-ordinate. Then, the total flux J_{st} is given by taking the equilibrium distribution function W'_{eq} for W' of Eq. (7):

$$J_{st} = J_{st}^{(1)} \int_{-\infty}^{+\infty} \dots \int_{-\infty}^{+\infty} W'_{eq}(q_2, q_3, \dots, q_f, p_2, p_3, \dots, p_f) dq_2 dq_3 \dots dq_f \times dp_2 dp_3 \dots dp_f, \quad (15a)$$

$$\text{with } W'_{eq} = N' \exp[-\{p_2^2/2 M_2 + p_3^2/2 M_3 + \dots + p_f^2/2 M_f + \tilde{U}(q_2, q_3, \dots, q_f)\}/kT], \quad (15b)$$

where N' is a normalization constant.

The number of systems in the vicinity of the equilibrium initial state is

$$n_i = N_i \int_{(q; \text{initial configurations})} \dots \int_{(p; -\infty}^{+\infty} \exp[-\{\sum_{\alpha=1}^f p_\alpha^2/2 M_\alpha + U(q_1, q_2, \dots, q_f)\}/kT] dq_1 dq_2 \dots dq_f dp_1 dp_2 \dots dp_f, \quad (16)$$

whose range of integration with respect to the position co-ordinates is limited to the configurations in the initial state, and N_i is a normalization constant.

The rate constant k_i for the chemical reaction from the initial state to the final state can be expressed in the following form,

$$k_i = J_{st}/n_i = \kappa(kT/h) \{Q_t/Q_i\} \exp(-U_t/kT), \quad (17a)$$

⁵ S. CHANDRASEKHAR, Rev. Mod. Phys. 15, 1 (1943).

where the transmission coefficient is defined by

$$\kappa = \{(\zeta_{11}/2 \omega_t M_1)^2 + 1\}^{1/2} - (\zeta_{11}/2 \omega_t M_1), \quad (17b)$$

and the partition functions are defined classically by

$$Q(t) = h^{-(f-1)} \int_{-\infty}^{+\infty} \cdots \int_{-\infty}^{+\infty} \exp \left[-\{p_2^2/2 M_2 + p_3^2/2 M_3 + \cdots + p_f^2/2 M_f + \tilde{U}(q_2, q_3, \dots, q_f)\}/kT \right] dq_2 dq_3 \dots dq_f dp_2 dp_3 \dots dp_f \quad (17c)$$

for the molecular configurations in the neighbourhood of the transition surface, and

$$Q(i) = h^{-f} \int_{(q: \text{initial configurations})} \cdots \int_{p; -\infty}^{+\infty} \exp \left[-\left\{ \sum_{\alpha=1}^f p_{\alpha}^2/2 M_{\alpha} + U(q_1, q_2, \dots, q_f) \right\}/kT \right] dq_1 dq_2 \dots dq_f dp_1 dp_2 \dots dp_f \quad (17d)$$

for the molecular configurations in the initial state, respectively. When the bottom of the potential hollow belonging to the initial state is chosen as the original level of the energy scale, Eq. (17a) can be transformed into

$$k_i = \kappa(kT/h) \{Q(t)/Q(i)\} \exp(-\Delta U_i/kT), \quad (17')$$

with $\Delta U_i = (U_t - U_i)$.

Here the value at the potential bottom of the initial state has been called U_i .

This expression can be compared with Eyring's one of rate constant. It must be remarked that the transmission coefficient is introduced inevitably to the expression of Eq. (17a), and has definite physical meaning.

For the case of large friction of

$$1/\tau_1 \equiv \zeta_{11}/M_1 \gg 2 \omega_t, \quad (18a)$$

where τ_1 is the time constant of the relaxation process of the momentum referring to the reaction co-ordinate, the transmission coefficient of Eq. (17b) is reduced to

$$\kappa \simeq (M_1 \omega_t / \zeta_{11}). \quad (18b)$$

On the other hand, for the frictionless case of the limit of

$$\zeta_{11} \rightarrow 0, \quad (19a)$$

the transmission coefficient approaches to unity,

$$\kappa \rightarrow 1. \quad (19b)$$

This last situation in the limit of Eq. (19a) corresponds correctly to Eyring's treatment.

It will be easily seen that the case of Eq. (18a) is equivalent to the treatment on the basis of the diffusion model of chemical reaction in a narrow sense. It has been known that KRAMERS-CHANDRASEKHAR's equation being the diffusion equation in phase space is transformed into Smoluchowski's diffusion equation in position space only, if we ignore all effects which occur in intervals of the order $\tau = M/\zeta$. In the present problem, by making use of the local equilibrium for the momentum distribution

$$W_1(q_1, p_1; t) \simeq w_1(q_1; t) (2\pi M_1 kT)^{-1/2} \exp(-p_1^2/2 M_1 kT), \quad (20)$$

and integrating suitably Eq. (8) with respect to p_1 , we obtain the following diffusion equation of Smoluchowski's,

$$\left. \begin{aligned} \partial w_1 / \partial t &= - \partial / \partial q_1 J_s^{(1)} \\ J_s^{(1)} &= - D^{(1)} \partial w_1 / \partial q_1 + (F_1 / \zeta_{11}) w_1 \end{aligned} \right\}, \quad (21)$$

where $J_s^{(1)}$ denotes to be Smoluchowski's diffusion flux

along the reaction co-ordinate q_1 , and the diffusion constant is defined by $D^{(1)} \equiv kT/\zeta_{11}$.

If the equilibrium condition is valid in the position space too, we obtain

$$w_1 = \text{constant} \times \exp \{-U(q_1, q_2, \dots, q_f)/D^{(1)}\zeta_{11}\} \quad (22a)$$

by the integration of Eq. (21) under the condition of $J_s^{(1)} = 0$.

Combining Eq. (22a) with Eq. (20), the following equilibrium distribution function can be obtained,

$$W_{1,eq} = N_{eq} \exp[-\{p_1^2/2 M_1 + U\}/kT], \quad (22b)$$

where N_{eq} is a normalization constant.

The expression of the rate constant on the basis of Eq. (21) under the steady condition is given by ^{6,7}

$$k_i^{(1)} = (kT/\zeta_{11}) \left\{ \int_{\text{range of potential barrier}} \exp(U/kT) dq_1 \int_{\text{initial configurations}} \exp(-U/kT) dq_1 \right\}^{-1}. \quad (23)$$

By making use of Eq. (4) for the potential energy in the neighbourhood of the transition surface, and supplementing the integration with regard to the space of momenta and the other co-ordinates, Eq. (23) becomes

$$\begin{aligned} k_i &= (kT/\zeta_{11} h) \left\{ \frac{\int_{-\infty}^{+\infty} \exp(-p_1^2/2 M_1 kT) dp_1}{\int_{-\infty}^{+\infty} \exp(-\omega_t^2 M_1 x^2/2 kT) dx} \right\} \times \\ &\times \{Q(t)/Q(i)\} \exp(-\Delta U_i/kT) = \\ &= (M_1 \omega_t / \zeta_{11}) (kT/h) \{Q(t)/Q(i)\} \exp(-\Delta U_i/kT), \end{aligned} \quad (24)$$

where $Q(t)$, $Q(i)$, and ΔU_i have the same forms and meanings as before. It has been found that Eq. (24) is just identical with Eq. (17') under the condition of Eq. (18a), and the transmission coefficient is given by

$$\kappa = M_1 \omega_t / \zeta_{11}.$$

Therefore, it is concluded that the rate constant of Eq. (17'), derived from the application of KRAMERS-CHANDRASEKHAR's equation to the motion of a single degree of freedom along the reaction co-ordinate, serves as a general expression which unifies the two types of effusion and diffusion of chemical reaction.

Zusammenfassung. Der bei Ablauf einer chemischen Reaktion stattfindende Elementarvorgang wird mit Hilfe des Diffusionsmodells untersucht. Der hierbei für die Geschwindigkeitskonstante gewonnene Ausdruck [Gl. (17')] stimmt in dem Grenzfalle, dass der Reibungskoeffizient verschwindet, mit dem Ergebnis nach der Eyringschen Übergangsmethode und im Grenzfalle grosser Reibungskoeffizienten mit der Darstellung Gleichung (24) für den Smoluchowskischen Diffusionsfluss im Lagekoordinatenraume überein.

N. TAKEYAMA⁸

The Institute of Biophysics, Faculty of Agriculture, Kyushu University, Fukuoka (Japan), April 17, 1961.

⁶ H. C. BRINKMAN, *Physica* 22, 29, 284 (1956).

⁷ J. L. WOOD and A. SUDDABY, *Trans. Faraday Soc.* 53, 1437 (1957).

⁸ The author takes great pleasure in acknowledging the encouragement of Prof. K. OOMORI.

6. Schweizerische elektronenmikroskopische Tagung

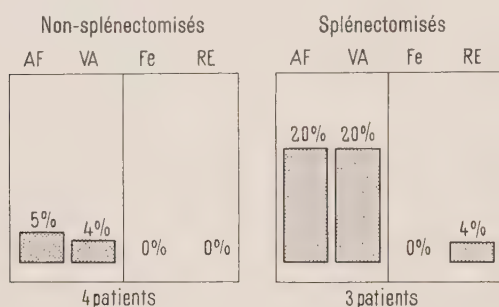
Anorganisch-chemisches Institut der Universität Bern,
15. Mai 1961

Originalmitteilungen

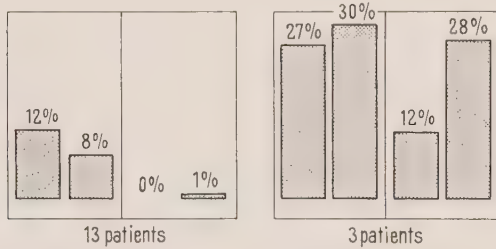
Étude systématique des altérations cellulaires au microscope électronique: application aux érythropathies

L'importance des altérations submorphologiques des érythrocytes du sang circulant de patients atteints de thalassémie n'est pas directement superposable à l'atteinte génétique: nous l'avons signalé avec MARINONE dès 1958¹⁻³. L'étude de ce phénomène a nécessité la mise au point d'une technique de routine permettant une analyse quantitative; dans ce but, les altérations observées ont été

Patients atteints de Thalassémie (minime, mineure ou majeure).



Sujets normaux et patients atteints d'affections diverses (mais pas de Thalassémie).



(Statistique portant sur 4754 érythrocytes)

Fréquence des altérations constatée dans les érythrocytes de 23 patients.

Thrombocytes et thrombopathies

L'étude submicroscopique systématique de thrombocytes provenant d'une quinzaine de patients atteints de diathèses hémorragiques nous a permis de faire certaines observations préliminaires.

Nous avons pu mettre en évidence une chute de nombre de *granulations denses* dans trois cas de déficience de la thromboplastine hématique, liée à l'activité anormalement faible du facteur 3 plaquettaire (une thrombopathie familiale¹, une thrombocytose hémorragique, un syndrome de Willebrand-Jürgens). Le nombre de ces granulations n'atteint plus que le tiers du nombre normal. Ces observations peuvent être considérées comme une confirmation des travaux de SCHULZ² et de FEISSLY et al.³ sur la localisation du facteur 3 plaquettaire dans ces organites.

Nous avons retrouvé les «granulations denses en baguettes de tambour» signalées par SCHULZ⁴ dans les

classées en 4 groupes principaux: *anomalies de forme* (AF), *vacuolisation* (VA), *amas de ferritine/hémossidérite* (Fe) et présence d'*organites d'origine érythroblastique* (RE). La fréquence de l'apparition de ces 4 groupes est notée sur des micrographies électroniques de coupes fines d'érythrocytes, puis exprimée en pour cent des éléments observés. Ainsi pouvons-nous établir une «formule» pour chaque patient.

Exemple: patient: G. d'I. 53 a.; Thalassémie mineure, splénectomisé depuis 19 a.; G.R.: $4,8 \cdot 10^6$; HbF: 8,3%.

«Formule»: AF = 36; VA = 32; Fe = 17; RE = 29.

Une publication ultérieure précisera cette technique, indiquera ses limites et discutera les résultats qu'elle permet d'obtenir. Son application à l'étude du sang de 23 sujets a donné les valeurs représentées schématiquement dans la Figure. Cette méthode bien qu'employée dans un faible nombre de cas s'est montrée utile et fournit des résultats reproductibles. Les valeurs portées sur la Figure confirment l'influence de la splénectomie sur la fréquence des altérations érythrocytaires déjà constatée par l'examen qualitatif de ces préparations. Elles livrent aussi d'intéressants renseignements sur les fonctions de normalisation de la rate et confirment ainsi les indications de CROSBY⁴. De plus, dans deux cas d'anémie hémolytique familiale non sphérocytaire⁵, l'utilisation de cette méthode a donné des informations concordant avec les données cliniques et les résultats des examens de laboratoire.

Summary. In order to attain a quantitative appraisal of cellular alterations, a statistical method has been developed. Preliminary results of its application to the study of pathological human erythrocytes are reported. They allow a correlation to the function of the spleen.

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avec la collaboration technique de
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¹ G. MARINONE et al., *Haematologica* 43, 1123 (1958).

² G. MARINONE, *Bull. Soc. vaud. Sci. nat.* 67, 109 (1959).

³ G. MARINONE et A. GAUTIER, in P. INTROZZI, *Trattato italiano di medicina interna* (Abruzzini Ed. Rome 1960).

⁴ W. H. CROSBY, *Blood* 14, 399 (1959).

⁵ O. TÖNZ et al., *Helv. paed. Acta*, 16, 111 (1961).

thrombocytes du patient atteint d'un syndrome de Willebrand-Jürgens ainsi que chez 4 patients atteints de thromboasthénie et dans un cas de réticulo-endothéliose avec anomalies plaquettaires. Nous ne pouvons donc considérer ces organites comme pathognomoniques d'une affection donnée.

Par l'analyse statistique des thrombocytes de 4 membres d'une même famille atteints de la thromboasthénie de Glanzmann-Naegeli^{5,6} nous avons pu mettre en évidence

¹ G. HEMMELER, *J. Suisse méd.* 41, 1018 (1958).

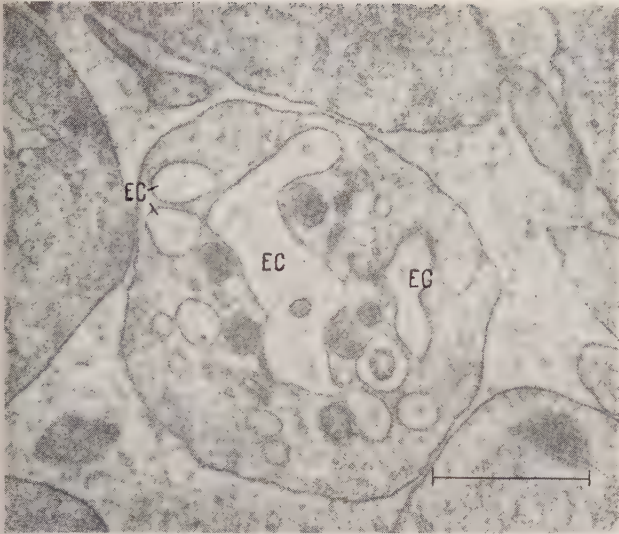
² H. SCHULZ et E. HIEPLER, *Klin. Wschr.* 37, 273 (1959).

³ R. FEISSLY et al., *Proc. 7th Congr. intern. Soc. Blood Transf.*, Rome 1958, 931 (1959).

⁴ H. SCHULZ et al., *Klin. Wschr.* 2, 300 (1958).

⁵ R. MARX et G. KÖPPEL, *Sangre* 2, 142 (1957).

⁶ Nous remercions le Professeur Dr. R. MARX de la 1^{re} Clinique médicale de l'Université de Munich qui a mis ce matériel à notre disposition.

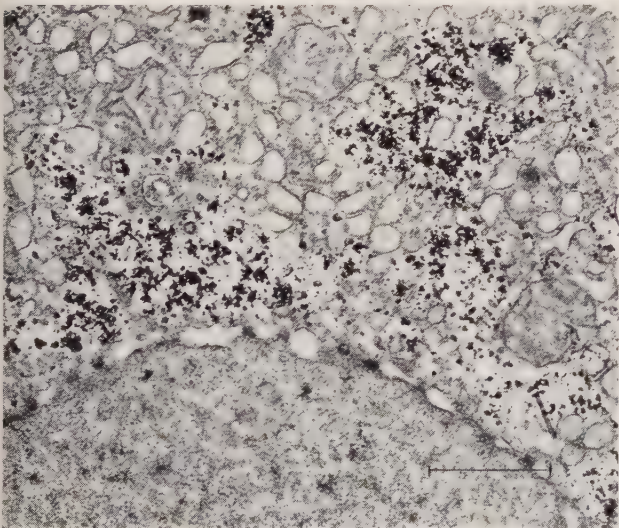


Thromboasthénie: l'aspect vacuolisé de ces thrombocytes est dû au développement anormal du complexe des éléments clairs (EC) du granulomère. Gross. $\times 20000$.

Cytochimie ultrastructurale. Fixations et colorations

Afin de préciser la base histochimique de certaines colorations utilisées actuellement en cytologie électronique, nous avons cherché à établir les effets d'éventuelles interactions entre colorants et agents fixateurs. Nous avons donc effectué différentes colorations sur des coupes ultrafines de tissus animaux fixés à l'acide osmique, au permanganate de potassium ou au formol, inclus dans divers polymères, après avoir soumis ou non ces coupes à une oxydation préalable par l'eau oxygénée ou par l'acide periodique.

Nos premiers résultats nous permettent de retenir les indications suivantes:



Mise en évidence du glycogène dans le cytoplasme d'une cellule hépatique. Fixation à l' OsO_4 tamponné selon Palade; inclusion au Vestopal W; oxydation de la coupe par H_2O_2 à 2% pendant 35 min; coloration pendant 35 min à 60°C par une solution d' AgNO_3 à 0,1%, alcalinisée à pH 8 par du borax à 5%. Gross. $\times 15000$.

4 types d'altération plaquettaire: une anisocytose notable, une vacuolisation importante due au développement anormal des *éléments clairs* (Figure), une réduction du nombre des *mitochondries*, parfois gonflées, et la présence de *stéatose*. Ces observations indiquent une altération profonde du métabolisme plaquettaire; on peut les mettre en rapport avec les analyses biochimiques de GROSS et al.⁷ Elles peuvent expliquer le défaut de rétractibilité du caillot qui caractérise la thromboasthénie⁸.

Summary. Preliminary results of a statistical survey of electron micrographs of thrombocytes from 15 patients with haemorrhagic diathesis are reported. The possible significance of ultrastructural changes in relation to factor 3 activity and metabolism deficiency are discussed.

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⁷ R. GROSS et al., Klin. Wschr. 38, 198 (1960).

⁸ Travail effectué avec l'appui du Fonds National Suisse de la Recherche Scientifique.

La présence d'*osmium réduit* influence d'une manière déterminante les résultats de la plupart des surcolorations et particulièrement celles faites au permanganate de potassium, à l'acétate de plomb et au nitrate d'argent.

L'*élimination* par l'eau oxygénée de l'*osmium réduit* inverse les contrastes de certains constituants cytoplasmiques (mais pas des ribosomes) dans le cas de tissus fixés à l'*osmium* et inclus au Vestopal W. Elle souligne l'affinité de l'*acétate de plomb* alcalinisé à la soude pour les nucléoprotéines en diminuant la colorabilité des autres constituants cellulaires; les résultats sont alors très semblables à ceux obtenus par le même colorant sur des tissus fixés au formol. La colorabilité par l'*acétate d'uranyle*, par contre, n'est pas modifiée par une oxydation préalable.

La coloration par le *nitrate d'argent alcalinisé au borax* après fixation osmique produit une coloration intense du *glycogène* (Figure) qui est indépendante d'une oxydation préalable à l'acide periodique. L'argentaffinité du *glycogène* résulte vraisemblablement de l'action réductrice des groupes aldéhydiques libérés par l'acide osmique^{1,2}; elle est inhibée par un traitement prolongé à l'eau oxygénée. La coloration des autres constituants cellulaires, par cette même solution de nitrate d'argent/borax, après élimination de l'*osmium réduit* ou après fixation au formol, est probablement due à la présence d'un composé d'argent soluble dans l'*hyposulfite de soude*.

Summary. First results of a systematic study of electron stainings are reported. These have been performed on osmium, permanganate or formol-fixed tissues, mainly embedded in polyester, with or without preliminary oxidization of the sections. An evaluation of the influence on the stainings of the tissue-bound osmium has been attempted. A new staining technique for glycogen is also described.

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¹ V. MARINOZZI, J. biophys. biochem. Cytol. 9, 121 (1961).

² M. WOLMAN, Exp. Cell Res. 12, 231 (1957).

Elektronenoptische Beobachtungen bei Speicherkrankheiten¹

Es wird über die Ultrastruktur der Speicherzellen in Milz und Leber bei je einem Fall von *Morbus Gaucher* und *Niemann-Pick* berichtet.

Die erhobenen Befunde lassen unter anderem folgende Feststellungen zu:

(1) Die elektronenmikroskopische Untersuchung erlaubt eine deutliche Unterscheidung dieser beiden Krankheitsbilder. Sie hat also diagnostischen Wert.

(2) Die Speicherzellen enthalten im Cytoplasma abnorme Gebilde, die als Speicherkörper bezeichnet werden. Diese zeigen eine unterschiedliche, wahrscheinlich mit der ungleichen chemischen Zusammensetzung in Beziehung stehende Ultrastruktur.

(3) Es finden sich Anhaltspunkte für die Annahme, dass die Speicherung im Bereich der Mitochondrien beginnt.

(4) In den Zellen mit abnormer Sphingomyelinspeicherung (Niemann-Picksche Krankheit) weisen *alle* Mitochondrien eine pathologische Struktur auf (Verkleinerung, diffus verstärkte Osmiophilie, undeutliche Membranzeichnung, knäuelartig angeordnete Cristae), auch diejenigen, die noch nicht speichern.

Die elektronenoptische Untersuchung vermag somit Störungen im Feinbau der Zellorganellen aufzudecken, die sich noch nicht im Endresultat der enzymatischen Fehlleistung, in diesem Fall der Speicherung, kundtun.

Summary. Ultrastructural changes in spleen and liver in a case of *Morbus Gaucher* and *Niemann-Pick* are discussed.

The results are: (1) The Ultrastructure shows a distinct differentiation between the two diseases. (2) It seems that storage begins in mitochondria. (3) In the case of *Morbus Niemann-Pick*, all Mitochondria of storage cells show an abnormal ultrastructure (diminution in size, increased osmiophilia, disorder in the arrangement of cristae and membranes).

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¹ Die Arbeit erscheint ausführlich an anderer Stelle.

Sur l'ultrastructure de l'appareil juxtaglomérulaire du rein

Dans la région du pôle vasculaire des corpuscules rénaux, on trouve, réunis sous le nom d'appareil juxtaglomérulaire, des dispositifs spéciaux, à savoir la macula densa, les cellules de Goormaghtigh, les cellules épithélioïdes du segment préglomérulaire de l'artériole afférente; certains auteurs y ajoutent les îlots cellulaires paraportaux de Becher. Nous avons étudié au microscope électronique (RCA, modèle EMU 3C) cette partie du rein de quelques mammifères (souris, rats, lapins, chat et bœuf), enrobé dans du méthacrylate et, plus souvent, dans du vestopal W, et avons obtenu les résultats que nous allons résumer¹.

Les cellules de la *macula densa* (plaque dense) montrent, dans leur partie basale, des cytomembranes modérément développées et assez irrégulièrement disposées, formant avec les loges cytoplasmiques intercalées un «labyrinthe basal» (Figure 1). Les mitochondries, localisées de préférence dans une situation circum- et supranucléaire, sont relativement petites et peu fréquentes. L'appareil de Golgi a une position infranucléaire. Ces caractéristiques permettent de distinguer les cellules maculaires des autres cellules de la partie contournée du segment intermédiaire.

¹ O. BUCHER et E. REALE, *Z. Zellforsch.* 54, 167 (1961); *Z. mikr.-anat. Forsch.*, 67, 514 (1961).

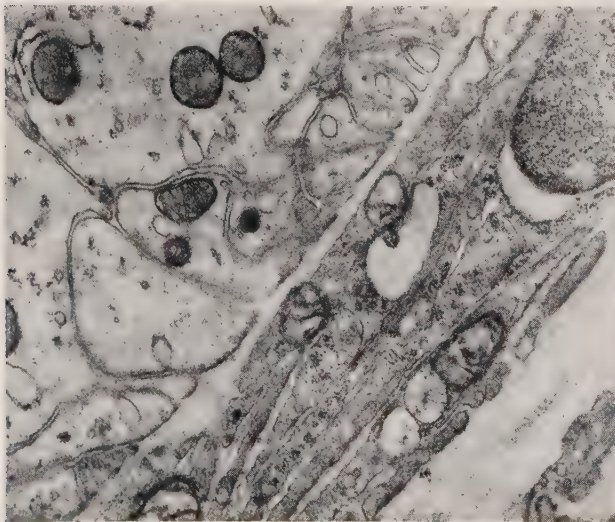


Fig. 1. En haut et à gauche, reposant sur une membrane basale, deux cellules d'une macula densa avec leur labyrinthe basal. En dessous, un groupe de cellules de Goormaghtigh, enchâssées dans un lacs de membranes intercellulaires, et ensuite la capsule d'un corpuscule rénal. En haut et à droite, un noyau de Goormaghtigh avec l'espace périnucléaire élargi. Rein de souris. Grossissement 19200 fois.



Fig. 2. A gauche, deux cellules épithélioïdes avec les caractéristiques grains de sécrétion. A droite, un corpuscule rénal. Rein de souris. Grossissement 8100 fois.

Les *cellules de Goormaghtigh*, situées à l'angle entre les artérioles afférente et efférente, sont en étroit rapport topographique avec les cellules épithélioïdes et avec la plaque dense, bien qu'en étant délimitées par une membrane basale. Elles sont enchâssées dans un système complexe de cloisons occasionnellement interrompues («lakis») ayant le caractère de membranes basales, positives à la réaction au PAS. Sous la surface irrégulière du noyau, il y a une accumulation de chromatine. L'espace péri-nucléaire est élargi dans les reins de souris fixés à la température ambiante à l' OsO_4 (Figure 1), mais pas après fixation à 0–4°C à l' OsO_4 additionné de sucrose. Dans le cytoplasme, on peut voir beaucoup de mitochondries, de vésicules et de ribosomes.

Les *cellules épithélioïdes* des artérioles afférentes sont également placées dans un cloisonnage de membranes basales dont l'épaisseur et la continuité sont irrégulières; les rapports entre les cellules sont très compliqués. Dans le cytoplasme, on remarque des granules (grains de sécrétion, Figure 2) dont l'abondance, la taille et la forme varient avec l'état fonctionnel. Ils sont entourés d'une membrane lisse. En outre, le corps cellulaire renferme des mitochondries, un appareil de Golgi et un ergastoplasme

bien développés, des ribosomes et parfois des faisceaux de fines fibrilles.

Une tentative d'interprétation du rôle physiologique de l'appareil juxtaglomérulaire en vertu des résultats ultrastructuraux acquis jusqu'à présent nous semble prématurée.

Summary. This paper deals with the description of the ultrastructure of the juxtaglomerular complex of the kidney, namely the epithelioid cells (polkissen) of the afferent arteriole, the Goormaghtigh cells, and the macula densa of the distal convoluted tubule, studied with the electron microscope. All these cells show a characteristic feature, different from that of all other kidney cells.

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² Ce travail a été effectué, en collaboration avec le Centre de Microscopie électronique de l'Université de Lausanne, dans le cadre de recherches subventionnées par la «Fondation Fritz Hoffmann-La Roche pour l'expansion, en Suisse, du travail scientifique exécuté par équipe».

Elektronenoptische Untersuchungen der Rattenleber nach chronischer Verabreichung von Äthyl- und Methylalkohol

Die krankhaften Veränderungen, die am menschlichen Organismus bei chronischem Alkoholabusus auftreten, haben seit Jahrzehnten die Medizin beschäftigt. Das meist umstrittene Organ hinsichtlich der schädigenden Wirkung des Alkohols ist die Leber. Dabei steht nicht zur Frage ob, sondern wie der Alkohol das Organ beeinflusst und zu krankhaften Veränderungen führt. Zur Klärung des Mechanismus der Entstehung der Lebercirrhose ist das Tierexperiment herangezogen worden. Die vorliegenden Untersuchungen beschäftigen sich mit den morphologischen Veränderungen an der Rattenleber bei chronischer Verabreichung von Äthyl- und Methylalkohol.

Material und Methode. Männliche weisse Ratten im Anfangsgewicht von 60–70 g wurden in Gruppen von je 3 Ratten unterteilt und erhielten ein vollwertiges Futter¹ und Trinklösung *ad libitum*. Bei den Kontrolltieren bestand das Trinkwasser aus Brunnenwasser, bei den Testtieren aus Brunnenwasser mit einem Zusatz von 10 Vol% Äthylalkohol bzw. 2,5 Vol% Methylalkohol. Nach 200 Tagen wurde der Versuch abgebrochen, die Ratten getötet.

Fixation in Osmiumsäure, Entwässerung und Einbettung in ein Methyl-*n*-Butylmetacrylatgemisch erfolgten nach den üblichen Methoden. Die Schnitte wurden mit dem Porter-Blum-Mikrotom mit mechanischem Vorschub hergestellt. Zur Beobachtung der Schnitte stand uns das Siemens-Elmiskop I mit einer Kathodenspannung von 60 kV zur Verfügung. Ausserdem wurden zur Vergleichsmöglichkeit sowohl in Paraffin eingebettete Leberstücke als auch dicke Schnitte aus den Metacrylatblöcken gefertigt und mit den üblichen Färbemethoden behandelt.

Resultate. Lichtmikroskopische Untersuchungen. Die optischmikroskopisch untersuchten Schnittserien von Kontroll- und Testratten weisen keine bedeutenden Unterschiede auf. Die Verfettung der Leberzellen von Äthylratten ist spärlich, die der Methylratten etwas ausgeprägter. In den ca. 1 μ dicken Schnitten aus den Metacrylatblöcken sieht man bei den Methylratten eine Vergrößerung der Feinstruktur des Protoplasma der Leberzellen. Dieses erscheint feinkörnig oder vacuolär. Anzeichen von Leber-

cirrhose mit grobem Umbau der Leberstruktur finden sich nirgends.

Elektronenoptische Untersuchungen. Die Beobachtungen stützen sich auf rund 500 elektronenmikroskopische Aufnahmen aus zahlreichen im Elektronenmikroskop durchgemusterten Schnitten.

Zellkerne. Die Untersuchung der Zellkerne bei Kontroll- und Testtieren zeigt keine Unterschiede. Kernmembran, Nucleoplasma und Nucleolus zeigen durchweg normale Struktur.

Endoplasmatisches Reticulum. Das endoplasmatische Reticulum, welches bei der normalen Rattenleber aus wechselnd dicht liegenden länglichen oder ovalen Bläschen und Schläuchen von zum Teil paralleler Anordnung besteht, zeigt bei den Äthanolratten keine oder höchstens diskrete, bei den Methanolratten schwerere Veränderungen.

Das endoplasmatische Reticulum der Äthanolratten ist sehr häufig insofern verändert, als das Zysternen- und Kanalsystem der Mikrosomen gequollen erscheint. Es ist ausserordentlich schwer, bei der Häufigkeit der Kunstprodukte, die bei der Metacrylateinbettung entstehen, abzuschätzen, ob die Quellungseffekte des endoplasmatischen Reticulums pathologischer Natur sind. Indessen treten diese Erscheinungen mit ziemlich häufiger Konstanz auf.

Die Veränderungen des endoplasmatischen Reticulums bei den Methanolratten sind in unserem Bildmaterial durchweg schwerer Natur. Die bei den Äthylratten (Figur 2) zum Teil nur andeutungsweise vorhandene Quellung des endoplasmatischen Reticulums ist hier hochgradig und nahezu in sämtlichen Bildern zu sehen. Die cytoplasmatische Matrix lässt sich kaum erkennen. Im ganzen Protoplasma finden sich hochgradig erweiterte, zum Teil stark verformte Vacuolen. Einige Vacuolen sind grösser als die dazwischenliegenden, oft stark komprimierten und verformten Mitochondrien. Die Begrenzung

¹ Die zu unserer Untersuchung verwendeten Leberstücke stammen von Ratten, die uns freundlicherweise vom medizinisch-chemischen Institut (Prof. Dr. H. AEBI und Dr. med. J. P. v. WARTBURG) zur Verfügung gestellt wurden.

Folgende Standard-Futtergemische wurden verwendet: (a) Nafag-Rattenwürfel (Mühle Landshut AG, Utzensdorf), (b) Brovo-Futter für Ratten, pulverisiert (Juramill AG, Laufen).

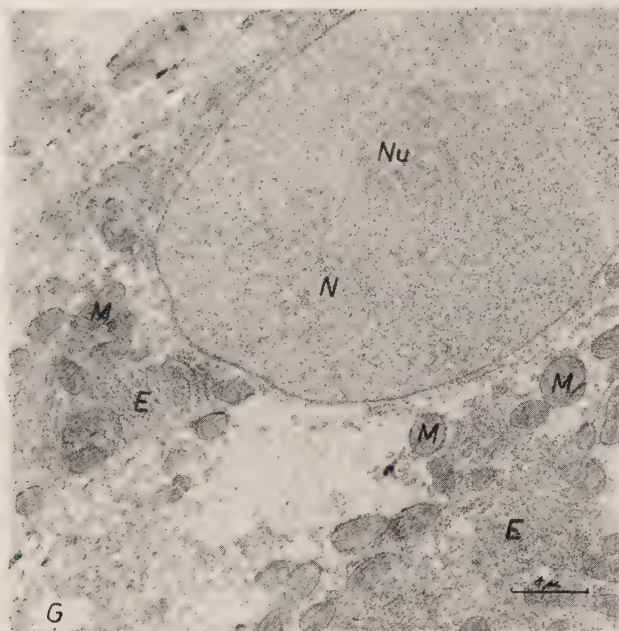


Fig. 1. Normale Rattenleberzelle mit Kern (N) und Nucleolus (Nu). Im Protoplasma die Mitochondrien (M) und das endoplasmatische Reticulum (E). In der linken untern Bildecke eine quergetroffene Gallenkapillare.

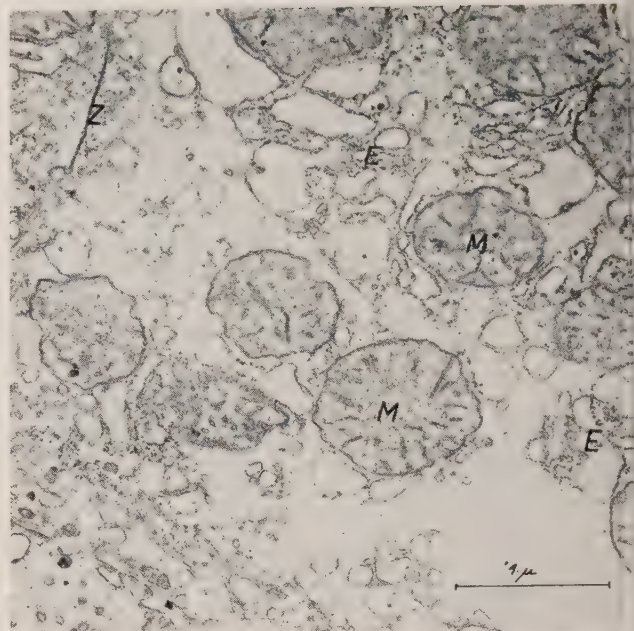


Fig. 2. Rattenleberzelle einer mit Äthanol belasteten Ratte. Endoplasmatisches Reticulum (E) gequollen. Mitochondrien (M) mit heller Matrix und radiär gestellten Cristae, geschwellt. Im oberen Bildrand links eine Zellmembran (Z).

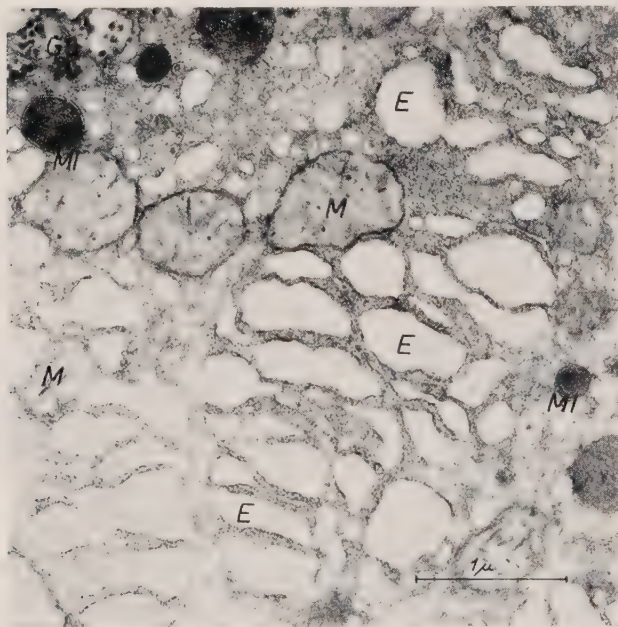


Fig. 3. Endoplasmatisches Reticulum (E) der mit Methylalkohol belasteten Ratten hochgradig geschwellt. Mitochondrien (M) komprimiert, zum Teil eingedellt. Vereinzelte Microbodies (MI). In der oberen linken Bildecke ein Golgiapparat.

der Vacuolen entspricht derjenigen der von PALADE beschriebenen «smooth»- oder «rough-surfaced»-Mikrosomen. Die oft deutlichen, zum Teil etwas vergrößerten deutlich sichtbaren Paladeschen Granula berechtigen uns anzunehmen, dass es sich um eine Quellung der oben erwähnten Mikrosomen handelt (Figur 3).

Mitochondrien. Ein statistisch signifikanter Unterschied zwischen den normalen Lebermitochondrien und denjenigen der Äthylratten lässt sich, in Bezug auf die

Grössendimension, nicht finden. Immerhin findet man mit derselben Häufigkeit wie das gequollene endoplasmatische Reticulum grosse geschwellte Mitochondrien mit heller Matrix und radiär angeordneter Cristae (Figur 2). Daneben findet man normal grosse und normal konfigurierte Mitochondrien. Pathologische Formen mit körnig zerfallener Feinstruktur sind keine seltenen Befunde, sind aber für die Äthylratten nicht typisch. Die Mitochondrien der Äthylratten liegen oft im geschwellten endoplasmatischen Reticulum (Figur 3) und sind hochgradig komprimiert, zum Teil eingedellt.

«Microbodies». «Microbodies» treten sowohl bei Kontroll- als auch bei Testtieren mit Regelmässigkeit auf. Ein vermehrtes Auftreten bei einer bestimmten Versuchsanordnung und somit eine Aussage über eine Regenerationsrate der Mitochondrien lässt sich nicht machen.

Golgi-Apparat. Veränderungen am Golgiapparat konnten weder an Kontroll- noch an Testtieren gefunden werden.

Zellmembran und Gallenkapillaren. An Zellmembranen und Gallenkapillaren können keine morphologischen Veränderungen gefunden werden. Insbesondere kann kein Auseinanderweichen der Zellmembranen beobachtet werden.

Summary. Male rats were fed *ad libitum*. As drinking solution they got instead of water a 10 vol% solution of ethanol or a 2.5 vol% solution of methanol. After 200 days all animals were sacrificed. Ultrastructure of liver cells was studied.

The morphological alterations in 'ethanol-rats' are slight or not existent. In 'methanol-rats' the endoplasmic reticulum is highly enlarged, the mitochondria small and compressed. Nucleus, Golgi complex, and plasma membrane do not show any pathological alteration.

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